

Xth International Symposium on Cholinergic Mechanisms

Arcachon, France, September 1-5, 1998

From *Torpedo* electric organ to human brain: fundamental and applied aspects

*De l'organe électrique de torpille
au cerveau humain :
aspects fondamentaux et appliqués*



EVIER

Edited by JEAN MASSOULIÉ

AD_____

AWARD NUMBER DAMD17-98-1-8635

TITLE: Tenth International Symposium on Cholinergic Mechanisms

PRINCIPAL INVESTIGATOR: Dr. Jean Massoulie

CONTRACTING ORGANIZATION: International Symposium Cholinergic Mech Lab
Neurobiologie CNRS
Paris Cedex France 05, 75230

REPORT DATE: January 1999

TYPE OF REPORT: Final Proceedings

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5102

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE January 1999	3. REPORT TYPE AND DATES COVERED Final (1 Jan 98 - 30 Sep 98)	
4. TITLE AND SUBTITLE Tenth International Symposium on Cholinergic Mechanisms			5. FUNDING NUMBERS DAMD17-98-1-8635	
6. AUTHOR(S) Dr. Jean Massoulie				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) International Symposium Cholinergic Mech Lab Neurobiologie CNRS Paris Cedex France 05, 75230			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words)				
14. SUBJECT TERMS Chemical Defense, Conference			15. NUMBER OF PAGES 466	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Xth International Symposium on Cholinergic Mechanisms

Arcachon, France, September 1-5, 1998

From *Torpedo* electric organ to human brain: fundamental and applied aspects

*De l'organe électrique de torpille
au cerveau humain :
aspects fondamentaux et appliqués*

Edited by Jean Massoulié



19990125 025



ELSEVIER

Paris, Amsterdam, Oxford, New York, Tokyo

23, rue Linois, 75724 Paris cedex 15

DTIC QUALITY INSPECTED 3

List of sponsors

Abbott (CH)	Fondation Simone et Cino del Duca (F)
ANPP, Association pour la Neuro-Psycho-Pharmacologie (F)	Hoffman-Laroche (CH)
AFM, Association Française contre les Myopathies (F)	INRA, Institut National de la Recherche Agronomique (F)
AFIRST, Association Franco-Israélienne pour la recherche scientifique et technique (F)	INSERM, Institut National de la Recherche Médicale (F)
BioLogic Claix (F)	ISN, International Society of Neurochemistry
Boehringer Ingelheim Italie (I)	Ministère des affaires étrangères (F)
CEA, Centre de l'Energie Atomique (F)	National Institutes of Health (USA)
CNRS, Centre National de la Recherche Scientifique (F)	Novartis (CH)
DGA, Direction Générale de l'Armement	Pharmacia-Upjohn (S)
EDF, Electricité de France (Service de radioprotection) (F)	Rhône-Poulenc-Rohrer (F)
EMBO, European Molecular Biology Organisation (EU)	Sanofi Recherche Montpellier (F)
EISAI/PFIZER	Société des Neurosciences (F)
FOA, Swedish Defence Research Establishment (S)	Synthélabo (F)
Fondation Ipsen (F)	United States Army Medical Defense Command (USA)
	Zeneca (GB)

Logo Xth ISCM by Jacqueline Chapron and Jean Massoulié

©1998 Éditions scientifiques et médicales Elsevier

Tous droits de traduction, d'adaptation et de reproduction par tous procédés réservés. En application de la loi du 11 mars 1957, il est interdit de reproduire, même partiellement, la présente publication sans l'autorisation de l'éditeur ou du Centre français du droit de copie (20, rue des Grands-Augustins, 75006 Paris).

All rights reserved. No part of this publication may be translated, reproduced, stored in a retrieval system or transmitted in any form or by other any means, electronic, mechanical, photocopying, recording or otherwise, without prior permission of the publisher.

Imprimé en France par l'imprimerie Louis-Jean, 05000 Gap.
Dépôt légal : 688 - Septembre 1998

ISBN : 2-84299-065-X

Foreword

The International Symposia on Cholinergic Mechanisms (ISCM) were established in Stockholm, Sweden, in 1970 and have been held every three years to discuss progress in the understanding of molecular, pharmacological, behavioural and clinical aspects of cholinergic mechanisms. The volumes published after each symposium have been landmarks in this field (the proceedings of the last two meetings were special issues of *Progress in Brain Research*). Following Sweden, Switzerland, USA, Italy, Great Britain, Canada and Germany, France hosts the Tenth ISCM in September 1998, in Arcachon. The marine laboratory in Arcachon has played an important role in cholinergic research since David Nachmansohn discovered there, in 1938, that the electric organs of *Torpedo* are a rich source of cholinergic synapses.

The meeting addresses cholinergic mechanisms in central and peripheral nervous systems, sensory organs and muscles. The development and the structure of the vertebrate neuromuscular junction are discussed, as well as its pathologies (myasthenia gravis and myasthenic syndromes). In the brain, cholinergic transmission or neuromodulation is involved in memory, sleep and arousal. Its dysfunction underlies nicotine addiction, as well as various neurological and psychiatric disorders such as dementia and some epilepsies. The molecular genetics of cholinergic mechanisms (nicotinic and muscarinic receptors, cholinesterases, acetylcholine synthesis and release) are progressing rapidly. Cholinergic drugs are being used or evaluated for treatment of myasthenia gravis and Alzheimer's disease. Cholinergic toxicology includes poisoning by organophosphate pesticides and nerve gases, with the intriguing Persian Gulf Syndrome. The Tenth ISCM in Arcachon is an opportunity to discuss exciting new insights into fundamental aspects of cholinergic mechanisms and their consequences for toxicology, pharmacology, medicine and the environment. This volume offers an up-to-date account of current research in the cholinergic field, from the molecular level to the most integrated levels, and its applications in toxicology and medicine.

Dr. Jean Massoulié
Chairman Organizing Committee

Organizing Committee

Yves Agid	(Paris, France)	Jeanine Koenig	(Bordeaux, France)
Daniel Bertrand	(Geneva, Switzerland)	Salomon Langer	(Paris, France)
Guy Blanchet	(Paris, France)	Jacques Mallet	(Paris, France)
Jean Cartaud	(Paris, France)	André Ménez	(Saclay, France)
Jean-Pierre Changeux	(Paris, France)	Nicolas Morel	(Gif-sur-Yvette, France)
Michel Fardeau	(Paris, France)	Jean-Pierre Toutant	(Montpellier, France)

International Advisory Committee

Marc Ballivet	(Geneva, Switzerland)	Alexander Karczmar	(Hines, USA)
René Couteaux	(Paris, France)	Konrad Löffelholz	(Mainz, Germany)
Claudio Cuello	(Montreal, Canada)	Marsel Mesulam	(Chicago, USA)
Ezio Giacobini	(Geneva, Switzerland)	Giancarlo Pepeu	(Florence, Italy)
Zach Hall	(Bethesda, USA)	Israel Silman	(Rehovot, Israel)
Edith Heilbronn	(Stockholm, Sweden)	Hermona Soreq	(Jerusalem, Israel)
Steve Heinemann	(San Diego, USA)	Palmer Taylor	(San Diego, USA)
Ferdinand Hucho	(Berlin, Germany)	Stanislav Tucek	(Prague, Czech Republic)
Maurice Israel	(Gif-sur-Yvette, France)	Victor P. Whittaker	(Cambridge, UK)

David
Nachmansohn



Arcachon, 1938

From left to right:
A. Chweitzer (*Paris*), W. Feldberg
(*Cambridge*), H. Blaschko
(*Cambridge*), R. Sigalas (*Directeur de
la station*), D. Nachmansohn (*New
York*) and A. Fessard (*Paris*).

George Koelle



La Grande-Motte, 1990.
From left to right: Miro Brzin, Winifred Koelle and George Koelle.

George Koelle



La Grande-Motte, 1990.
From left to right: Miro Brzin, Winifred Koelle and George Koelle.

Xth International Symposium on Cholinergic Mechanisms

Coordinated by Jean Massoulié, Paris, France

Arcachon and cholinergic transmission <i>V.P. Whittaker (Göttingen, Germany)</i>	53
Early days in the research to localize skeletal muscle acetylcholinesterases <i>R. Couteaux (Paris, France)</i>	59

David Nachmansohn Lecture

Allosteric nicotinic receptors, human pathologies <i>C. Léna, J.-P. Changeux (Paris, France)</i>	63
---	----

George B. Koelle Lecture

Dissection of active zones at the neuromuscular junction by EM tomography <i>M. Harlow, D. Ress, B. Koster, R.M. Marshall, M. Schwarz, U.J. McMahan (Stanford, USA; Martinsried, Germany)</i>	75
--	----

Molecular organization of the nicotinic receptors

Toxins selective for subunit interfaces as probes of nicotinic acetylcholine receptor structure <i>P. Taylor, H. Osaka, B.E. Molles, N. Sugiyama, P. Marchot, E.J. Ackermann, S. Malany, J.J. McArdle, S.M. Sine, I. Tsigelny (San Diego, La Jolla, Newark, Rochester, USA)</i>	79
The role of subunit interfaces for the nicotinic acetylcholine receptor's allosterism <i>F. Hucho, C. Methfessel, A. Watty (Berlin, Leverkusen, Germany)</i>	85
Allosteric modulation of neuronal nicotinic acetylcholine receptors <i>B. Buisson, D. Bertrand (Geneva, Switzerland)</i>	89
Molecular dissection of subunit interfaces in the nicotinic acetylcholine receptor <i>S.M. Sine, N. Bren, P.A. Quiram (Rochester, USA)</i>	101
Functional determinants by which snake and cone snail toxins block the $\alpha 7$ neuronal nicotinic acetylcholine receptors <i>D. Servent, H. Lam Thanh, S. Antil, D. Bertrand, P.J. Corringer, J.P. Changeux, A. Ménez (Gif-sur-Yvette, Paris, France; Geneva, Switzerland)</i>	107
Congenital myasthenic syndromes: Experiments of nature <i>A.G. Engel, K. Ohno, S.M. Sine (Rochester, USA)</i>	113

Molecular mechanisms of acetylcholine release: the choline acetylcholinetransferase and acetylcholine transporter gene locus

Regulated exocytosis in neurons and neurosecretory cells: Structural events and expression competence <i>J. Meldolesi (Milan, Italy)</i>	119
Acetylcholine release. Reconstitution of the elementary quantal mechanism <i>M. Israël, Y. Dunant (Gif-sur-Yvette, France; Geneva, Switzerland)</i>	123
Protein interactions implicated in neurotransmitter release <i>O. El Far, V. O'Connor, T. Dresbach, L. Pellegrini, W. DeBello, F. Schweizer, G. Augustine, C. Heuss, T. Schäfer, M.P. Charlton, H. Betz (Frankfurt, Germany; Durham, USA; Basel, Switzerland; Toronto, Canada)</i>	129
On the action of botulinum neurotoxins A and E at cholinergic terminals <i>P. Washbourne, R. Pellizzari, O. Rossetto, N. Bortoletto, V. Tugnoli, D. De Grandis, R. Eleopra, C. Montecucco (Padova, Ferrara, Italy)</i>	135
Dissociation of the vesicular acetylcholine transporter domains important for high-affinity transport recognition, binding of vesamicol and targeting to synaptic vesicles <i>H. Varoqui, J.D. Erickson (New Orleans, USA)</i>	141
The cholinergic locus: ChAT and VACht genes <i>J. Mallet, L. Houhou, F. Pajak, Y. Oda, R. Cervini, S. Bejanin, S. Berrard (Paris, Evry, France)</i>	145
Regulation of the cholinergic gene locus <i>H. Tanaka, M. Shimojo, D. Wu, L.B. Hersh (Lexington, USA)</i>	149

Supramolecular organization and pathology of the neuromuscular junction

The Schwann cell at the neuromuscular junction <i>J. Koenig, S. De La Porte, J. Chapron (Paris, Talence Gif-sur-Yvette, France)</i>	153
Lipid peroxidation and changes in cytochrome c oxidase and xanthine oxidase activity in organophosphorus anticholinesterase induced myopathy <i>Z.P. Yang, W.-D. Dettbarn (Nashville, USA)</i>	157
Regulation of gene expression by trans-synaptic activity: A role for the transcription factor NF- κ B <i>V. Gisiger (Montreal, Canada)</i>	163
Development of the neuromuscular junction: Genetic analysis in mice <i>J.R. Sanes, E.D. Apel, R.W. Burgess, R.B. Emeson, G.Feng, M. Gautam, D. Glass, R.M. Grady, E. Krejci, J.W. Lichtman, J.T. Lu, J. Massoulié, J.H. Miner, L.M. Moscoso, Q. Nguyen, M. Nichol, P.G. Noakes, B.L. Patton, Y.-J. Son, G.D. Yancopoulos, H. Zhou (St. Louis, Nashville, Tarrytown, USA; Paris, France)</i>	167
Transcriptional pathways for synapse-specific, neuregulin-induced and electrical activity-dependent transcription <i>L. Fromm, S.J. Burden (New York, USA)</i>	173
Targeting of acetylcholine receptor and 43 kDa rapsyn to the postsynaptic membrane in <i>Torpedo marmorata</i> electrocyte <i>F. Bignami, G. Camus, S. Marchand, L. Bailly, F. Stetzkowski-Marden, J. Cartaud (Paris, France)</i>	177
Acetylcholinesterase: C-terminal domains, molecular forms and functional localization <i>J. Massoulié, A. Anselmet, S. Bon, E. Krejci, C. Legay, N. Morel, S. Simon (Paris, France)</i>	183
Three-dimensional structure of a complex of E2020 with acetylcholinesterase from <i>Torpedo californica</i> <i>G. Kryger, I. Silman, J.L. Sussman (Rehovoth, Israel; Upton, USA)</i>	191
Targeting acetylcholinesterase molecules to the neuromuscular synapse <i>R.L. Rotundo, S.G. Rossi, H.B. Peng (Miami, Chapel Hill, USA)</i>	195

Central cholinergic mechanisms; nicotinic receptors and nicotine dependence

Imprinting of hippocampal metabolism of choline by its availability during gestation: Implications for cholinergic neurotransmission <i>J.K. Blusztajn, J.M. Cermak, T. Holler, D.A. Jackson (Boston, USA)</i>	199
TrkA antagonists decrease NGF-induced ChAT activity in vitro and modulate cholinergic synaptic number in vivo <i>T. Debeir, H. Uri Saragovi, A.C. Cuello (Montreal, Canada)</i>	205
Nicotine modifies the activity of ventral tegmental area dopaminergic neurons and hippocampal GABAergic neurons <i>J.L. Fisher, V.I. Pidoplichko, J.A. Dani (Houston, USA)</i>	209
The hypothesis of an ambient level of acetylcholine in the central nervous system <i>L. Descarries (Montreal, Canada)</i>	215
The role of neuronal nicotinic acetylcholine receptors in antinociception: Effects of ABT-594 <i>M.W. Decker, P. Curzon, M.W. Holladay, A.L. Nikkel, R. Scott Bitner, A.W. Bannon, D.L. Donnelly-Roberts, P.S. Puttfarcken, T.A. Kuntzweiler, C.A. Briggs, M. Williams, S.P. Arneric (Abbott Park, USA)</i>	221
Neural substrate of nicotine addiction as defined by functional brain maps of gene expression <i>E. Merlo Pich, C. Chiamulera, M. Tessari (Verona, Italy)</i>	225
Is dopamine important in nicotine dependence? <i>M. Shoaib (London, UK)</i>	229

Development of cholinergic systems; muscarinic receptors

Brain choline has a typical precursor profile <i>K. Löffelholz</i>	235
Positive effects of allosteric modulators on the binding properties and the function of muscarinic acetylcholine receptors <i>S. Tucek, J. Jakubík, V. Dolezal, E.E. El-Fakahany (Prague, Czech Republic; Minneapolis, USA)</i>	241

On the transcriptional regulation of neuronal nAChR genes <i>J.-M. Matter, L. Matter-Sadzinski, T. Roztocil, M.-C. Hernandez, S. Couturier, M.-T. Ong, M. Ballivet (Geneva, Switzerland)</i>	245
Position effect variegations and brain-specific silencing in transgenic mice overexpressing human acetylcholinesterase variants <i>M. Sternfeld, J.D. Patrick, H. Soreq (Jerusalem, Israel; Houston, USA)</i>	249
Muscarinic acetylcholine receptors activate the acetylcholinesterase gene promoter <i>R.M. Nitsch, S. Rossner, C. Albrecht, M. Mayhaus, J. Enderich, M. Wegner, R. Schliebs, T. Arendt, H. von der Kammer (Hamburg, Leipzig, Germany)</i>	257
Structure-function analysis of muscarinic acetylcholine receptors <i>E. Kostenis, F.-Y. Zeng, J. Wess (Bethesda, USA)</i>	265
Scanning mutagenesis of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor <i>E.C. Hulme, Z.-L. Lu (London, UK)</i>	269
Molecular mechanisms for the regulation of the expression and function of muscarinic acetylcholine receptors <i>S.E. Hamilton, M.L. Schlador, L.A. McKinnon, R.S. Chmelar, N.M. Nathanson (Seattle, USA)</i>	275

Cholinergic neurons: pathologies and therapeutic strategies in motoneuron disease and Alzheimer's disease

Role of neurotrophic factors in motoneuron development <i>C.E. Henderson, Y. Yamamoto, J. Livet, V. Arce, A. Garces, O. deLapeyrière (Marseille, France)</i>	279
Cholinergic foundations of Alzheimer's disease therapy <i>E. Giacobini (Geneva, Switzerland)</i>	283
The role of A β 42 in Alzheimer's disease <i>S.G. Younkin (Jacksonville, USA)</i>	289
Some cholinergic themes related to Alzheimer's disease: Synaptology of the nucleus basalis, location of m2 receptors, interactions with amyloid metabolism, and perturbations of cortical plasticity <i>M.-M. Mesulam (Chicago, USA)</i>	293
The effects of the acetylcholinesterase inhibitor ENA713 and the M1 agonist AF150(S) on apolipoprotein E deficient mice <i>S. Chapman, A. Fisher, M. Weinstock, R. Brandies, E. Shohami, D.M. Michaelson (Tel-Aviv, Ness-Ziona, Jerusalem, Israel)</i>	299

Cholinergic toxicology: acute and subacute exposure to cholinesterase inhibitors. Past experience and future implications

Neuromuscular responses to pyridostigmine bromide in organotypic spinal cord-muscle culture <i>R. Drake-Baumann, F.J. Seil, P.S. Spencer (Portland, USA)</i>	305
Contribution of nicotinic receptors to the function of synapses in the central nervous system: The action of choline as a selective agonist of $\alpha 7$ receptors <i>E.X. Albuquerque, E.F.R. Pereira, M.F.M. Braga, M. Alkondon (Baltimore, USA; Rio de Janeiro, Brazil)</i>	309
Chronic neurobehavioral and central and autonomic nervous system effects of Tokyo subway sarin poisoning <i>K. Yokoyama, S. Araki, K. Murata, M. Nishikitani, T. Okumura, S. Ishimatsu, N. Takasu (Tokyo, Japan)</i>	317
Long term health effects of low dose exposure to nerve agent <i>D.H. Moore (Bel Air, USA)</i>	325
Cholinergic excitation induces activity-dependent electrophysiological and transcriptional responses in hippocampal slices <i>A. Friedman, D. Kaufer, L. Pavlovsky, H. Soreq (Beersheva, Jerusalem, Israel)</i>	329

Round table: Cholinergic therapies of Alzheimer's disease

Novel m1 muscarinic agonists in treatment and delaying the progression of Alzheimer's disease: An unifying hypothesis <i>A. Fisher, R. Brandeis, R. Haring, N. Eshhar, E. Heldman, Y. Karton, O. Eisenberg, H. Meshulam, D. Marciano, Z. Pittel (Ness-Ziona, Israel)</i>	337
--	-----

Molecular interactions of acetylcholinesterase with senile plaques <i>N.C. Inestrosa, R. Alarcón (Santiago, Chile)</i>	341
---	-----

Round table: Regulation of the cholinergic system by other neurotransmitters

Cholinergic modulation of extracellular ATP-induced cytoplasmic calcium concentrations in cochlear outer hair cells. <i>M.A. Wikström, G. Lawoko, E. Heilbronn (Stockholm, Sweden)</i>	345
The acetylcholine, GABA, glutamate triangle in the rat forebrain <i>G. Pepeu, P. Blandina (Florence, Italy)</i>	351

Round table: Toxicology of anticholinesterase agents

Enzymes hydrolyzing organophosphates as potential catalytic scavengers against organophosphate poisoning <i>P. Masson, D. Josse, O. Lockridge, N. Vigué, C. Taupin, C. Buhler</i> <i>(La Tronche, France; Omaha, USA)</i>	357
Four acetylcholinesterase genes in the nematode <i>Caenorhabditis elegans</i> <i>M. Arpagaus, D. Combes, E. Culetto, M. Grauso, Y. Fedon, R. Romani, J.-P. Toutant</i> <i>(Montpellier, Antibes, France; Perugia, Italy)</i>	363
Medical management of organophosphate-induced seizures <i>G. Lallement, F. Dorandeu, P. Filliat, P. Carpentier, V. Baille, G. Blanchet</i> <i>(La Tronche, Paris, France)</i>	369
Treatment of organophosphate poisoning. Experience of nerve agents and acute pesticide poisoning on the effects of oximes <i>M. Balali-Mood, M. Shariat (Mashhad, Iran)</i>	375

Round table: To be or not to be a functional cholinergic cell

Influence of retinoic acid and of cyclic AMP on the expression of choline acetyltransferase and of vesicular acetylcholine transporter in NG108-15 cells <i>M.-F. Diebler, M. Tomasi, F.-M. Meunier, M. Israël, V. Dolezal</i> <i>(Gif-sur-Yvette, France)</i>	379
From the cholinergic gene locus to the cholinergic neuron <i>E. Weihe, M.K.-H. Schäfer, B. Schütz, M. Anlauf, C. Depboylu, C. Brett, L. Chen,</i> <i>L.E. Eiden (Marburg, Germany; Bethesda, USA)</i>	385
A syntaxin-SNAP 25-VAMP complex is formed without docking of synaptic vesicles <i>N. Morel, P. Taubenblatt, M. Synguelakis, G. Shiff (Gif-sur-Yvette, Paris, France)</i>	389

Concluding remarks

Conclusions and comments. Xth International Symposium on Cholinergic Mechanisms <i>A.G. Karczmar (Hines, Maywood, USA)</i>	393
Abstracts	401
Author index	513
List of participants	517

Arcachon and cholinergic transmission

Victor P. Whittaker*

Max-Planck-Institut für biophysikalische Chemie, D-37070 Göttingen, Germany

Abstract — The cholinergic nature of transmission at the electromotor synapse of *Torpedo marmorata* was established at Arcachon in 1939 by Feldberg, Fessard and Nachmansohn (*J. Physiol. (Lond.)* 97 (1939/1940) 3P–4P) soon after transmission at the neuromuscular junction had been shown to be cholinergic. In 1964, after a quarter of a century of neglect, workers in Cambridge, then in Paris, Göttingen and elsewhere, began to use this system, 500–1000 times richer in cholinergic synapses than muscle, for intensive studies of cholinergic transmission at the cellular and molecular level. (©Elsevier, Paris)

Resumé — Arcachon et la transmission cholinergique. La nature cholinergique de la transmission à la synapse électromotrice de la torpille a été établie à Arcachon en 1939 par Feldberg, Fessard et Nachmansohn (*J. Physiol. (Lond.)* 97 (1939/1940) 3P–4P), peu après la découverte du caractère cholinergique de la transmission à la jonction neuromusculaire. Après une période assez longue de désuétude, les chercheurs de Cambridge, Paris, Göttingen et d'ailleurs ont commencé, vers 1964, à utiliser ce système, 500–1000 fois plus riche en terminaisons cholinergiques que le muscle, pour étudier intensivement la transmission cholinergique au niveau cellulaire et moléculaire. (©Elsevier, Paris)

Torpedo marmorata / electric organ / electromotor synapse / cholinergic transmission

1. Introduction

The electric ray, *Torpedo marmorata*, is fairly common in the Bay of Biscay (Baie de Gascogne): although a slow breeder (6–12 young every 2 years), it is not fished commercially. Its chief interest for the neurobiologist is that it possesses paired electric organs on each side of the midline which are capable of giving shocks (up to 40 V in air), strong enough to stun prey and deter predators. These organs are under the control of the electromotor nerves which originate in the electric lobes, paired nuclei on the brain stem placed caudally to the cerebellum. The electromotor neurons are in turn under the control of the oval nucleus in the brain-stem. This integrates sensations of hunger, detection of prey and nociceptive stimuli and decides whether to release a train of electric shocks (short trains at a frequency of 100–300 Hz). The electric organ consists of stacks of flattened cells known as electrocytes, electroplaques or electropilax; these are densely innervated on their ventral surfaces by fine branches of the electromotor axons.

Torpedo spp. are not the only fish to have electric organs; members of at least seven other diverse families have acquired them by a process of convergent evolution (for a recent review see [21]).

However, in terms of availability, amount of tissue per specimen and density of synaptic material, the Torpedinidae, especially *T. marmorata* (Eastern Atlantic), *T. ocellata* (Mediterranean) and *T. californica* (Pacific), are the family of choice.

Electric organs have excited the interest of biologists since earliest times: the puzzling nature of their discharge was only resolved with the discovery of electricity and indeed led to the invention of the battery or Voltaic pile which A. Volta (for attributions in this form, see Whittaker [20] for original references) himself described, in a memoir to the Royal Society of London published in 1800, as an artificial electric organ. J. Bernstein correctly postulated in 1912 that the discharge was caused by a transient increase in the permeability of the electroplaque membrane to positively charged ions.

It has been known since the 1870s, mainly from the work of A. Babuchin in 1876, that the electrocytes are derived embryonically from myoblasts; it is therefore not surprising that the electromotor synapse is cholinergic. Grundfest [5] in a definitive review, reinterpreted Bernstein's theory of the discharge in the light of contemporary knowledge of the excitation of muscle; he described it as the summation in series and in parallel of normal excitatory postsynaptic potentials (EPSPs) evoked by the release of transmitter from the presynaptic nerve terminals. Unlike some other electric organs, the electrocytes of Torpedinidae are electrically inexcitable; thus the discharge in this type of electric organ is

*Address for correspondence: 197 Huntingdon Road, Cambridge CB3 0DL, UK

pure summed EPSP unmodified by a conducted response corresponding to a muscle action potential.

Apart from a few autonomic vascular endings, the innervation of the *Torpedo* electric organ is purely cholinergic with a synaptic content 500–1000 times that of muscle; indeed the organ resembles a huge mass of hypertrophied motor endplates. With 400–500 g of tissue per fish to work with, the electric organ is an ideal resource for studies of the cellular and molecular biology of all aspects of cholinergic transmission: the synthesis and storage of transmitter, its release and postsynaptic action, its hydrolysis to inactive products (choline and acetate) and their eventual reutilization. The electric lobes, though available in smaller quantities (ca. 400 mg/specimen), contain large (~40 μ m diameter) cholinergic cell bodies studded with putative glutamergic nerve terminals; they contain the complete genome for cholinergic function and the mechanisms for the formation and transport of synaptic vesicles. In only one important respect does the electromotor terminal differ from terminals in muscle (including *Torpedo* muscle): its synaptic vesicles are larger (~90 nm in diameter versus ~50 nm in muscle). This facilitates a study of their function which appears to be similar in all respects to that of their smaller congeners.

2. Discovery of the cholinergic nature of transmission at the electromotor synapse

The discovery that transmission at the electromotor synapse is cholinergic was made during 3 weeks of research in June 1939 as the coming World War cast its shadow over Europe. Three neurobiologists of very different backgrounds came together at the Station Biologique d'Arcachon where *Torpedo* from the Bassin was readily available. They were A. Fessard, an electrophysiologist from the Collège de France, Paris, D. Nachmansohn, a biochemist and German Jewish emigrant working at the Sorbonne, Paris, and W. Feldberg, a pharmacologist and also a German Jewish emigrant working with H.H. Dale in London.

Fessard had worked extensively with electric tissue (for literature citations see Feldberg and Fessard [4]). He showed that excitation could only be brought about via the nerve – denervated tissue was unexcitable mechanically and electrically – and that nerve action could be blocked by drugs (curare, eserine) that block the cholinergic transmission in muscle discovered by H.H. Dale, W. Feldberg and M. Vogt in 1936. He surmised that the discharge must be triggered by the release of a depolarizing

substance from the electromotor nerve terminals, probably acetylcholine, as in muscle.

Nachmansohn, a medical graduate of Berlin University, had received his early research training with O. Meyerhof, who had done classical work on the energy-yielding metabolic reactions sustaining muscular contractions. Nachmansohn thought that biochemical methods should be applied to nerve activity and argued that if acetylcholine were indeed a transmitter at the neuromuscular junction, the released transmitter must be destroyed by cholinesterase within the refractory period of muscle. With his student-assistant Annette Marnay he showed that the cholinesterase activity of the innervated portion of the frog sartorius muscle was several thousand times greater than that of the non-innervated portion and great enough to destroy released acetylcholine within the muscle's refractory period. Being aware that the electrocytes of electric tissue resembled motor endplates, he asked Marnay to test for the presence of cholinesterase in electric tissue. She found very high levels of activity in this tissue, exceeding that of muscle several hundred-fold.

Fessard heard about these results and invited Nachmansohn to work with him at Arcachon on chemical transmission in the electric organ. However, if the techniques the Dale group had used so successfully with muscle were to be applied to the electric organ, a third collaborator who could assay acetylcholine in tissue and perfusates and apply it to the organ by close arterial injection would be needed.

Accounts differ [20] on how Feldberg came to be invited to join Fessard and Nachmansohn but no-one better qualified than he could have been chosen, in view of his participation in the work on muscle.

The results showed unequivocally that transmission at the electromotor synapse is cholinergic. Publication was delayed by the outbreak of the Second World War in September 1939 and Nachmansohn's hasty departure for America, but the results were eventually written up by Feldberg and were published, together with some additional work Feldberg did in London on extracts of electric organ he brought back with him, in a now classical paper in the *Journal of Physiology* [4].

Three lines of evidence justify the conclusion that transmission at the electromotor synapse is cholinergic. Firstly, extracts of electric organ under conditions which prevent the breakdown of acetylcholine contain a substance which precisely matches the action of acetylcholine in a variety of assay systems: the frog rectus abdominis muscle; the dorsal muscle of the leech; slowing of the frog's heart; a fall in the cat's blood pressure; adrenaline release from the adrenal medulla. No other known substance would

match acetylcholine in all of them. The substance behaved like acetylcholine in its lability in alkaline solutions and its sensitivity to cholinesterase. The acetylcholine equivalence of the substance ($400 \text{ nmol g of tissue}^{-1}$) is within the range found by later authors. In the second line of evidence it is shown that what we can now assume to be acetylcholine is released into perfusates of the organ on stimulation and in the third, close arterial injection of authentic acetylcholine excited discharges similar to those evoked by nerve stimulation.

Some years later Woodin (cited by Whittaker [19]) confirmed the identity of the acetylcholine-like substance in electric organ as acetylcholine by paper chromatography and more recently, it has been unequivocally identified by gas chromatography-mass spectrometry [18].

Nachmansohn continued to work on electric organs in the US, but switched to the electric eel, *Gymnotus electricus*, a denizen of the Amazon. His views on the role of acetylcholine in the nervous system began to deviate sharply from those of Dale and most other neurobiologists (for a critique and references, see Whittaker [20]); nevertheless, his group made many important contributions to cholinergic neurobiology. The comparative electrophysiology of electric organs attracted sporadic attention during the 1950s and with the coming of electron microscopy, the fine structure of the electromotor synapse was studied in several Torpedinidae: *Narcine* [17]; *T. marmorata* [9, 13]. Nevertheless, the potentialities of the electromotor system for the investigation of the cellular and molecular biology of cholinergic transmission remained largely neglected for 25 years.

3. The electromotor innervation of *Torpedo* as a model cholinergic system

3.1. The electromotor synapse: cytoplasmic and vesicular pools of acetylcholine

My own involvement with the *Torpedo* electric organ started in 1963 soon after my discovery of the synaptosome and the successful isolation from synaptosomes of pure preparations of mammalian cortical synaptic vesicles, a proportion of which stored acetylcholine in amounts consistent with their being the morphological basis of quantal transmitter release [24, 26]. In 1963 a colleague, R.D. Keynes, suggested that *Torpedo* electric organ might be a better source of cholinergic vesicles than mammalian brain. The tissue proved difficult to homogenize, but with M.N. Sheridan and M. Israël [14, 27] synaptosomes in low yield and reasonably homogeneous

preparations of synaptic vesicles were obtained. Purer preparations of the latter were subsequently obtained by M. Israël on his return to France. Meanwhile, the large-scale isolation of pure synaptic vesicles was made possible [25] by three technical innovations: comminution of the tissue by crushing after rendering it brittle by freezing it at low temperatures; extraction of the crushed tissue with solutions iso-osmotic to *Torpedo* fluids; and high-resolution separation of the resultant cytoplasmic extracts on continuous density gradients in a large-capacity zonal rotor. This enabled the composition, biophysics and recycling of synaptic vesicles to be intensively studied [20]. Briefly, it was established by the Göttingen group that acetylcholine exists in two pools: the cytoplasmic, where it is synthesized, and the vesicular, in which it is stored and from which it is released. Small differences in density and composition enabled three pools of vesicles to be separated: largely empty vesicles newly arrived from the cell body by fast axonal transport [6], a reserve pool and a stimulus-induced recycling pool [29] which preferentially takes up acetylcholine newly synthesized in the cytoplasm. By using choline analogues it was possible to label the cytoplasmic and the two transmitter-containing vesicular pools differentially and to show that transmitter released by stimulation arises exclusively from the recycling pool [7].

The vesicular uptake of acetylcholine is driven by a proton gradient generated by a vacuolar-type ATPase [28] and is facilitated by a vesicular acetylcholine transporter (vAChT), the sequence of which is encoded within the first intron of the gene locus for choline acetyltransferase [3].

3.2. Synaptosomes and presynaptic plasma membranes

Improved methods of preparing synaptosomes were developed both by our group and that of Israël; when well sealed these were able to develop membrane potentials comparable to those in intact neurons [10, 11]. The specificity and properties of the choline uptake system were extensively studied [2, 23]. The plasma membranes are rich in a family of minor gangliosides containing a sialylated *N*-acetylgalactosamine residue; these are specific for mammalian cholinergic neurons and have permitted the immunoisolation of cholinergic synaptosomes from a mixed population (for review see [22]).

3.3. Electromotor cell bodies and axons

The cell bodies of electromotor neurons are $\sim 40 \mu\text{m}$ in diameter and thus among the largest ver-

tebrate motor neurons known. They are readily isolated and though shorn of their dendrites are well sealed. Their choline acetyltransferase activity is twice that of cholinergic cell bodies in *Aplysia* and about 40 times greater than mammalian ganglion cells, but only one quarter that of electromotor nerve terminals.

The perikarya of these cells are rich in Golgi membranes and synaptic vesicles and contain readily translatable mRNA which code inter alia for several synaptic vesicle proteins [12]. Choline acetyltransferase is transported down the axon at the slow rate [1]; synaptic vesicles are transported at the fast rate and take up acetylcholine only when they reach the terminal [6].

The nerve terminals in the lobe are those of afferents from the command or oval nucleus. They are probably glutamergic and have been isolated in low yield by conventional methods.

3.4. Cotransmission in the electromotor synapse

Many cholinergic neurons are now known to utilize various neuropeptides as co-transmitters. The electromotor neurons are no exception; as in mammalian brain and autonomic ganglia, VIP is the co-transmitter. It is transported to the terminal in dense-cored vesicles (for references see Whittaker [20]). Another vesicular constituent which may act as a cotransmitter or regulator is ATP, present in vesicles in a 1:5 ratio to acetylcholine.

3.5. The nicotinic acetylcholine receptor (nAChR)

In one of the major advances of modern neurobiology the acetylcholine receptor in the muscle and electrocyte postsynaptic membrane is now known to be a ligand-gated pentameric ion channel composed of four polypeptide subunits, α , β , γ , δ of known sequences in which the α unit is represented twice. Early work was done on *Electrophorus* (*Gymnotus*) electric organ but when it was realised (from about 1975) that *Torpedo* membranes are much richer in receptor than *Electrophorus*, there was a shift to *Torpedo* spp. (*T. californica* and *T. marmorata*) by the many groups working in this field. The complete receptor is a rivet-like structure which undergoes a tilting conformational change as it opens and closes [16].

3.6. Acetylcholinesterase

The type of cholinesterase present in electrocytes hydrolyses acetylcholine faster than other choline esters and is thus known as an acetylcholinesterase. It has been fully sequenced and its tertiary structure

and mode of catalysis are known. The choline moiety of acetylcholine is firmly held in a trough lined with 14 aromatic residues whose π electrons interact with the positive charge on the quaternary nitrogen of acetylcholine. Hydrolysis is effected by a charge relay system; the catalytic triad has been identified as Glu-327, His-440 and Ser-200. These widely separated residues are brought together by folding [15].

Massoulié and coworkers (for review see Massoulié and Toutant [8]) have shown that acetylcholinesterase can exist in several forms in electric organ and other tissues. The catalytic subunit (G_1) is a highly glycosylated soluble globular protein of molecular mass 70–85 kDa. In tissues a tetramer (G_4) of this unit is normally conjugated through S-S bonds to a collagen tail giving an asymmetric form (A_4). Further aggregation via interaction of the tails gives larger forms culminating in A_{12} , an aggregate of three A_4 units. Such forms are extractable by solutions of high ionic strength. In another conjugate, dimeric catalytic units are linked through ethanolamine, glycan and glucosamine to phosphatidylinositol (PI); this form is inserted via the lipid end of its tail into lipoprotein membranes. Such tails are found in several unrelated membrane-bound molecules with the common property of being solubilized by PI-specific phospholipase C (PIPLC).

4. The development of the electromotor system

This has been extensively studied by J. Mellinger in Rheims and by the Göttingen group (for references see Whittaker [20]). Nineteenth-century observations have been confirmed and extended by electronmicroscopic, electrophysiological and biochemical techniques. Briefly, four stages in development have been recognized: I, myogenic; II, electrocytogenic; III, synaptogenic divided into IIIa, penetration of stacks of electrocytes by ingrowing neurites, and IIIb, functional synaptogenesis. During phases II and IIIa, extensive apoptosis of electromotor neurons takes place as neurites are matched with their targets. Two trophic factors have been identified: a heat-stable neuronotrophic factor essential for neuronal survival and a heat-sensitive cholinotrophic factor that stimulates the expression of the cholinergic phenotype. The molecular structure of these factors has not been identified.

5. Conclusion

Although a vast amount of important work has been done with the electromotor system of *Torpedo*

since its cholinergic character was first established by Feldberg, Fessard and Nachmansohn, its potentiality is far from exhausted. While fish may not be so convenient to work with as small rodents, any difficulties in transporting and keeping them can be overcome by careful organization.

'Cholinologists' have reason to be grateful to the Station Biologique d'Arcachon for their exceptional willingness to provide *Torpedo* alive or as frozen tissues to so many laboratories and for the local organization and facilities which have made this possible.

References

- [1] Davies L.P., ATP in cholinergic nerves: evidence for the axonal transport of a stable pool, *Exp. Brain Res.* 33 (1978) 149–157.
- [2] Ducis I., Whittaker V.P., High-affinity sodium-gradient-dependent transport of choline into vesiculated presynaptic plasma membrane fragments from the electric organ of *Torpedo marmorata* and reconstitution of the solubilized transporter into liposomes, *Biochim. Biophys. Acta* 815 (1985) 109–127.
- [3] Erickson J.D., Varoqui H., Schäfer M.K.H., Modi W., Diebler M.F., Weihe E., Rand J., Eiden L.E., Bonner T., Usdin T.B., Functional identification of a vesicular acetylcholine transporter and its expression from a 'cholinergic' gene locus, *J. Biol. Chem.* 269 (1994) 21929–21932.
- [4] Feldberg W., Fessard A., The cholinergic nature of the nerves to the electric organ of the *Torpedo* (*Torpedo marmorata*), *J. Physiol.* 101 (1942) 200–216.
- [5] Grundfest H., The mechanism of discharge of the electric organ in relation to general and comparative electrophysiology, *Prog. Biophys. Chem.* 7 (1957) 1–85.
- [6] Kiene M.L., Stadler H., Synaptic vesicles in electromotoneurons. I. Axonal transport, site of transmitter uptake and processing of a core proteoglycan during maturation, *EMBO J.* 6 (1987) 2209–2215.
- [7] Luqmani Y.A., Sudlow G., Whittaker V.P., Homocholine and acetylhomocholine: false transmitters in the cholinergic electromotor system of *Torpedo*, *Neuroscience* 5 (1980) 153–160.
- [8] Massoulié J., Toutant J.P., Vertebrate cholinesterases: structure and types of interaction, in: Whittaker V.P. (Ed.), *The Cholinergic Synapse*, Springer Verlag, Berlin, 1988, pp. 167–224.
- [9] Mellinger J., Belbenoit P., Ravaille M., Szabo T., Electric organ development in *Torpedo marmorata*, *Chondrichthyes*, *Dev. Biol.* 67 (1978) 167–188.
- [10] Meunier F.M., Relationship between presynaptic membrane potential and acetylcholine release in synaptosomes from *Torpedo* electric organ, *J. Physiol.* 354 (1984) 121–137.
- [11] Richardson P.J., Whittaker V.P., The Na⁺ and K⁺ content of isolated *Torpedo* synaptosomes and its effect on choline uptake, *J. Neurochem.* 36 (1981) 1536–1542.
- [12] Schmid D., Stadler H., Whittaker V.P., The isolation, from electromotor neuron perikarya, of messenger RNAs coding for synaptic proteins, *Eur. J. Biochem.* 122 (1982) 633–639.
- [13] Sheridan M.N., The fine structure of the electric organ of *Torpedo marmorata*, *J. Cell Biol.* 24 (1965) 129–141.
- [14] Sheridan M.N., Whittaker V.P., Isolated synaptic vesicles: morphology and acetylcholine content, *J. Physiol.* 175 (1964) 25P–26P.
- [15] Sussman J.L., Harel M., Frolow F., Oefner C., Goldman A., Tokar L., Silman I., Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein, *Science* 253 (1991) 872–879.
- [16] Unwin P.N.T., Toyoshima C., Kubalek E., Arrangement of the acetylcholine receptor subunits in the resting and desensitized states, determined by cryoelectron microscopy of crystallized *Torpedo* postsynaptic membranes, *J. Cell Biol.* 107 (1988) 1123–1138.
- [17] Wachtel A., Mathewson R., Grundfest H., Electron microscopic and histochemical comparison of the two types of electroplaques of *Narcine brasiliensis*, *J. Biophys. Biochem. Cytol.* 11 (1961) 663.
- [18] Weiler M., Roed I.S., Whittaker V.P., The kinetics of acetylcholine turnover in a resting cholinergic nerve terminal and the magnitude of the cytoplasmic compartment, *J. Neurochem.* 38, (1982) 1187–1191.
- [19] Whittaker V.P., Identification of acetylcholine and related esters of biological origin, in: Koelle G.B. (Ed.), *Cholinesterases and Anticholinesterase Agents*, Springer Verlag, Berlin, 1963, pp. 1–39.
- [20] Whittaker V.P., *The Cholinergic Neuron and its Target: The Electromotor Innervation of the Electric Ray Torpedo as a Model*, Birkhäuser Verlag, Boston MA, 1992.
- [21] Whittaker V.P., Electric organs and their innervation: a model system for the study of cholinergic function, in: Walz D., Berg H., Milazzo G. (Eds.), *Bioelectrochemistry of Cells and Tissues*, vol 2, Birkhäuser Verlag, Basel, 1995, pp. 1–33.
- [22] Whittaker V.P., Kelić S., Cholinergic-specific glycoconjugates, *Neurochem. Res.* 20 (1966) 1377–1387.
- [23] Whittaker V.P., Luqmani Y.A., False transmitters in the cholinergic system: implications for the vesicle theory of transmitter storage and release, *Gen. Pharmacol.* 11 (1980) 7–14.
- [24] Whittaker V.P., Sheridan M.N., The morphology and acetylcholine content of isolated cerebral cortical synaptic vesicles, *J. Neurochem.* 12 (1965) 363–372.
- [25] Whittaker V.P., Essman W.B., Dowe G.H.C., The isolation of pure cholinergic synaptic vesicles from the electric organs of elasmobranch fish of the family Torpedinidae, *Biochem. J.* 128 (1972) 883–846.
- [26] Whittaker V.P., Michaelson I.A., Kirkland R.J.A., The separation of synaptic vesicles from nerve ending particles ('synaptosomes'), *Biochem. J.* 90 (1964) 293–305.
- [27] Whittaker V.P., Sheridan M.N., Israël M., The subcellular fractionation of the electric organ of *Torpedo*, *Z. Zellforsch.* 74 (1966) 291–307.
- [28] Yamagata S.K., Parsons S.M., Purification and subunit composition of a cholinergic synaptic vesicle glycoprotein, phosphointermediate-forming ATPase, *J. Neurochem.* 53 (1989) 1345–1353.
- [29] Zimmermann H., Denston C.R., Separation of synaptic vesicles of different functional states from the cholinergic synapses of the *Torpedo* electric organ, *Neuroscience* 2 (1977) 715–730.

Early days in the research to localize skeletal muscle acetylcholinesterases

René Couteaux

University Pierre-et-Marie-Curie, Institute of Neurosciences, 7, quai Saint-Bernard, 75006 Paris, France

Abstract — Very early in the study of the mechanism of neuromuscular transmission in skeletal muscles, it was clear that the hydrolysis of acetylcholine by muscle cholinesterases within the time of the refractory period required a very high concentration of the enzyme near the motor terminals. David Nachmansohn and George B. Koelle and their collaborators obtained the first biochemical and histochemical data consistent with this prediction. Now that the various molecular forms of AChE have been satisfactorily described, it is possible to analyse the mechanisms by which they are anchored to the structures of the neuromuscular junction, and in particular, to the synaptic basal lamina. (©Elsevier, Paris)

Résumé — Les premières étapes des recherches dans la localisation des acétylcholinestérases du muscle squelettique. En étudiant le mécanisme de la transmission neuromusculaire des muscles squelettiques, il est très tôt apparu que l'hydrolyse de l'acétylcholine par les cholinestérases musculaires dans les délais de la période réfractaire impliquait l'existence d'une très forte concentration enzymatique au voisinage immédiat des terminaisons motrices. C'est à David Nachmansohn et à George B. Koelle, ainsi qu'à leurs collaborateurs, que nous devons les premières données biochimiques et histochimiques qui s'accordent pleinement avec cette prévision. Maintenant que les diverses formes moléculaires de l'AChE ont été définies de manière satisfaisante, il est devenu possible d'entreprendre l'étude des modalités de leur ancrage dans les structures de la jonction neuromusculaire et, en particulier, dans la lame basale synaptique. (©Elsevier, Paris)

acetylcholinesterases / neuromuscular junction / synaptic transmission

Cholinesterases were among the first proteins to be studied when investigations of the mechanism of synaptic transmission began. Their localization and their function were lively debated between partisans and opponents of a 'neurohumoral' model of transmission at the neuromuscular junction of skeletal muscle.

As Dale first indicated in 1914, if acetylcholine is a major agent of synaptic transmission, it must be rapidly inactivated, presumably by an enzyme. Brown, Dale and Feldberg [3] believed that the elimination of ester liberated by stimulation associated with such neuromuscular transmission must occur with a 'flashlike' suddenness, within the limits of the refractory period, i.e., within one or a few ms. To explain such a rapid hydrolysis, they suggested that the esterase must be concentrated at the motor nerve endings. In 1936, this was only a hypothesis, which many physiologists and biochemists still considered highly unlikely. David Nachmansohn and George B. Koelle, who will be honoured at the next Arcachon Symposium, made decisive contributions to the validation of this hypothesis.

For the occasion of this Symposium on cholinergic mechanisms, where there will be frequent reference to the work of Nachmansohn and of Koelle, it seems to me to be appropriate to return, briefly, to these early days in the search to identify the sites of muscular cholinesterases, in which both were

outstanding figures. My own research on skeletal muscle repeatedly benefited from my direct and personal contact with these two pioneers in neurobiology, and particularly from my collaboration with Nachmansohn during his stay in France, at the Sorbonne, which was interrupted by the war.

This retrospective of what may seem to be prehistoric research can also be justified in view of the world events which often disrupted the publication and discussion of their results, as was also the case for work on the cholinesterases of the *Torpedo* electric organ, one of the central themes of the Arcachon Symposium of 1939.

David Nachmansohn and his student, Annette Marnay, first used the frog sartorius muscle to study the distribution of muscle cholinesterases. This muscle contains regions rich in motor nerve terminals, usually called 'neural' parts of the muscle, and regions poor in such terminals, called 'aneural' parts (Keith Lucas, 1907; Pezard and May, 1937). They hoped to find significantly different rates of hydrolysis of acetylcholine in the neural and aneural regions. The first results obtained by Marnay and Nachmansohn [17], presented to the meeting of the Physiological Society in London in February 1937, were disappointing. A difference of only 20% to 40% was found between neural and aneural regions. These values appeared too low to be considered evidence of a particular concentration of the enzyme

at motor nerve endings. In addition, when the rates of acetylcholine hydrolysis in the tissues of various guinea-pig organs were determined, the cholinesterase activities were not very different from those in skeletal muscle tissue. New experiments with the frog sartorius, but using homogenized samples rather than small pieces of muscle, were more conclusive [18, 19, 21]. Acetylcholine hydrolysis was three to four times higher in neural than aneural samples prepared in this way. In view of the extremely small volume of neuromuscular junctions, these observations were consistent with a very high concentration of cholinesterase in each junction. At the same time, Marnay and Nachmansohn performed analogous experiments with the dog gastrocnemius. This was a muscle whose motor innervation I had previously studied and so I could supply Marnay and Nachmansohn with neural and aneural part of this muscle. Despite only few assays, the difference between these two preparations of dog muscle was concordant with that found between analogous preparations of the frog sartorius.

A further work with the internal section of the guinea-pig gastrocnemius confirmed clearly these first results and allowed the conclusions drawn from the studies of frog end-bushes to be extended to mammalian motor end-plates [4, 9, 10, 11]. By measuring cholinesterase activity in a series of slices obtained with a freezing microtome with a suitable orientation, the distribution of the enzyme activity could be plotted and compared to that of the motor end-plates. The correlation between these two distributions was also established by treating alternate slices from the same muscle for biochemical assay of enzyme activity and staining to reveal nerve endings.

The high cholinesterase activity at the neuromuscular junctions was initially attributed to the nerve terminals themselves. The best way to confirm this appeared to be to study the changes in this activity following section of the motor nerve, during the subsequent loss of nerve endings. The first studies of denervated muscle cholinesterases were conducted by Martini and Torda [22] in an attempt to explain the hypersensitivity of denervated muscle to acetylcholine. Two weeks after sectioning the motor nerve of the rat gastrocnemius, they observed a loss of enzyme activity, which fell to half its normal value. At first view, these findings suggested that the cholinesterase at the junctions was mainly located in the nerve endings themselves.

To elucidate the role of acetylcholine in neuromuscular transmission, it was obviously important to determine whether the enzyme was indeed in the nerve endings of the junctions, or elsewhere. If acetylcholine were the neurotransmitter, it would have

to act and be inactivated outside the nerve endings. Repeating this denervation experiment with the guinea-pig gastrocnemius, Marnay and Nachmansohn [20] unexpectedly found an increase in the concentration of cholinesterase in the weeks following section of the motor nerve and degeneration of the axons.

To explain this increased concentration in guinea-pig muscles, Marnay and Nachmansohn [20] at first suggested that it might be linked to the increase in the number of nuclei observed following denervation (T. Cahn, 1927; S. Tower, 1935). Work on the development of the muscles of the hind leg of the chick embryo led Nachmansohn [25, 26] however to abandon his first hypothesis. In the muscles of the chick embryo, the cholinesterase concentration increases progressively during incubation, and then declines after hatching so that after 3 weeks the concentration is only 10% of that on the day of hatching. Recent publications concerning the formation of mammalian motor end-plates suggested to Nachmansohn an interpretation of these changes in cholinesterase concentration. To quote: 'the observations of Couteaux indicate that the end-plates reach nearly their final size at birth, so that while the muscle fibres of an embryo are small, the end-plates in a given weight of muscle are relatively both large and numerous' [26]. Variations in enzyme concentration could thus be explained to a large extent by changes in the ratio of the volume of the motor end-plates to that of the muscle fibres and consequently by differences in the number of motor end-plates per unit weight. The decrease in concentration following hatching would thus be due mostly to the increase in size of the muscle fibres.

By analogy, for muscles which become smaller following denervation (as in the case for the guinea-pig gastrocnemius) the variations in the concentration of enzyme following denervation were suggested to be mainly due to changes in the muscle fibre volume (*figure 1*). Two weeks after sectioning the sciatic nerve, the change in the concentration of the enzymatic activity was indeed found to be inversely proportional to the change in volume of the denervated muscle fibre: the former increasing while the latter decreased. Thus, the activity at each motor end-plate was almost unchanged, and the high concentration of cholinesterase at the normal neuromuscular junction was thus not in the nerve ending itself but outside and around it [4, 9, 10, 11].

This interpretation was satisfactorily consistent with findings for muscles in which the fibres decrease in volume only slowly after denervation. For example, in the toad sartorius (Feng and Ting, 1938) and the rat gastrocnemius (Stoerk and Morpeth, 1944) denervation does not lead to a rapid change

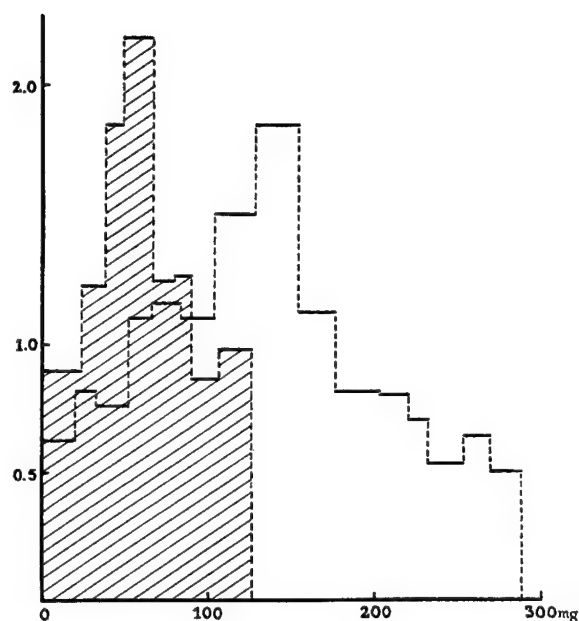


Figure 1. Concentration of cholinesterase activity in the middle portion ('neural' part) of the internal sections of two gastrocnemius muscles of the same guinea-pig. Each muscle was cut in consecutive slices and each horizontal line corresponds to the weight of a slice. Ordinates: cholinesterase activity (QChE). One of the muscles (hatched zone) was denervated by section of the sciatic nerve 16 days before. The comparison of the curves shows the persistence of a high enzymatic activity in the region of denervated endplates.

in the volume of the muscle fibres and thus not to a correlative increase in the number of denervated end-plates per unit weight. In both these muscles, the degeneration of motor axons is not followed by an increase in the concentration of the cholinesterase, but indeed by a decrease in its activity, most of which is expressed at the denervated end-plates.

The accumulation of biochemical data and the progress of morphological studies of the neuromuscular junction led to the suggestion some years later that the high cholinesterase activity of the neuromuscular junction was mainly located in the sub-neural or sub-synaptic apparatus [5]. Such an assumption could only be verified by histochemistry. Two years after it was formulated, Koelle and Friedenwald [16], using acetylthiocholine as the substrate, developed an appropriate histochemical technique. The images of sites of high concentration obtained by this approach, as viewed with the light microscope, were largely superimposable on those of the subneural apparatus obtained with certain aniline stains. This technique also revealed another, sometimes very ma-

jor, site of cholinesterase concentration at muscle-tendon junctions [7, 8]. The muscle fibre at this junction, where the sarcolemma presents folds comparable in certain respects to the subsynaptic folds, is more sensitive than in non-synaptic regions to acetylcholine [14].

Using selective inhibitors in association, this acetylthiocholine technique was highly specific, and could distinguish between acetylcholinesterases and other cholinesterases. However, the significance of the locations identified was more debatable. The technique could lead to major artefacts, particularly resulting from diffusion, which could lead to incorrect conclusions as to the site of the enzymatic activity. In the case of skeletal muscles, the reaction products (and not the enzymes) could diffuse to neighbouring sites. Various modifications of the technique were used and substantially reduced the extent of this problem [6, 12, 15]. Nevertheless, these artefacts limit the accuracy of the localization particularly as concerns sites which are close together, as in the case for the neuromuscular junction, the presynaptic plasma membrane, the synaptic basal lamina (BL), the postsynaptic plasma membrane and the Schwann cells.

Despite the use of numerous new histochemical techniques derived from the acetylthiocholine method, or using other substrates, doubts remained about the interpretation of the locations observed, in particular for the postsynaptic plasma membrane and the synaptic BL in the synaptic cleft. Correlations were established between the effects of the action of proteolytic enzymes on the synaptic BL and variations in the intensity of cholinesterase staining of end-plates. They suggested a link between the enzyme and synaptic BL [1, 2, 13]. This association between the junctional cholinesterase and the synaptic BL was demonstrated histochemically by McMahen, Sanes and Marshall [24] on the cutaneous pectoris muscle of the frog in which the neuromuscular junctions were deprived *in vivo* of their cellular components (nerve terminal, Schwann cell and muscle cell): only subsisted in the preparation the sheaths of BL which surrounded the muscle fibres. Using this preparation, histochemical staining revealed the persistence of the enzymatic activity in the synaptic BL. The reduction in staining intensity following inhibition by a specific inhibitor of 'true AChE' demonstrated that 'some if not all' of the cholinesterase in the BL was AChE.

To characterise the nature of the association of AChE with the synaptic BL and other muscle sites, it was necessary to investigate its molecular forms. Such studies showed that there were multiple forms of AChE with diverse quaternary structures and solubility characteristics [23]. The means of anchorage

of various asymmetric and globular AChE have been investigated in detail, and the results will be discussed during the Arcachon Symposium.

References

- [1] Betz W., Sakmann B., 'Disjunction' of frog neuromuscular synapses by treatment with proteolytic enzymes, *Nature (New Biol.)* 232 (1971) 94-95.
- [2] Betz W., Sakmann B., Effects of proteolytic enzymes on function and structure of frog neuromuscular junctions, *J. Physiol. (Lond.)* 230 (1973) 673-688.
- [3] Brown G.L., Dale H.H., Feldberg W., Reactions of the normal mammalian muscle to acetylcholine and eserine, *J. Physiol. (Lond.)* 87 (1936) 394-424.
- [4] Couteaux R., La cholinestérase des plaques motrices après section du nerf moteur, *Bull. Biol. France Belgique* 76 (1942) 14-57.
- [5] Couteaux R., Contribution à l'étude de la synapse myoneurale, *Rev. Canad. Biol.* 6 (1947) 563-711.
- [6] Couteaux R., Remarques sur les méthodes actuelles de détection histochimique des activités cholinestérasiques, *Arch. Int. Physiol.* 59 (1951) 52-63.
- [7] Couteaux R., Particularités histochimiques des zones d'insertion du muscle strié, *C.R. Soc. Biol. (Paris)* 147 (1953) 1974-1976.
- [8] Couteaux R., The differentiation of synaptic areas (Lecture), *Proc. Roy. Soc. (London)* B 158 (1963) 457-480.
- [9] Couteaux R., Nachmansohn D., Cholinesterase at the endplates of voluntary muscles after nerve degeneration, *Nature* 142 (1938) 481.
- [10] Couteaux R., Nachmansohn D., La cholinestérase des plaques motrices, *Bull. Soc. Chim. Biol.* 21 (1939) 1054-1055.
- [11] Couteaux R., Nachmansohn D., Changes of cholinesterase at end-plates of voluntary muscles following section of sciatic nerve, *Proc. Soc. Exp. Biol. Med.* 43 (1940) 177-181.
- [12] Couteaux R., Taxi J., Recherches histochimiques sur la distribution des activités cholinestérasiques au niveau de la synapse myoneurale, *Arch. Anat. Micr.* 41 (1952) 352-392.
- [13] Hall Z.W., Kelly R.B., Enzymatic detachment of endplate acetylcholinesterase from muscle, *Nature (New Biol.)* 232 (1971) 62-63.
- [14] Katz B., Miledi R., The development of acetylcholine sensitivity in nerve-free muscle segments, *J. Physiol. (Lond.)* 156 (1961) 24 p.
- [15] Koelle G.B., The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions, *J. Pharmacol. Exp. Ther.* 103 (1951) 153-171.
- [16] Koelle G.B., Friedenwald J.S., A histochemical method for localizing cholinesterase activity, *Proc. Soc. Exp. Biol. Med.* 70 (1949) 617-622.
- [17] Marnay A., Nachmansohn D., Choline esterase in voluntary frog's muscle, *J. Physiol. (Lond.)* 89 (1937) 359-371.
- [18] Marnay A., Nachmansohn D., Cholinestérase dans le muscle strié, *C.R. Soc. Biol. (Paris)* 124 (1937) 942-944.
- [19] Marnay A., Nachmansohn D., Sur la répartition de la cholinestérase dans le muscle couturier de la grenouille, *C.R. Soc. Biol. (Paris)* 125 (1937) 41-43.
- [20] Marnay A., Nachmansohn D., Cholinestérase dans le muscle strié après dégénérescence du nerf moteur, *C.R. Soc. Biol. (Paris)* 126 (1937) 785-787.
- [21] Marnay A., Nachmansohn D., Choline esterase in voluntary muscle, *J. Physiol. (Lond.)* 92 (1938) 37-47.
- [22] Martini E., Torda K., Die Cholinesterase des denervierten Muskels, *Klin. Wochenschrift* 16 (1937) 41-43.
- [23] Massoulié J., Bon S., The molecular forms of cholinesterase and acetylcholinesterase in vertebrates, *Annu. Rev. Neurosci.* 5 (1982) 57-106.
- [24] McMahan U.J., Sanes J.R., Marshall L.M., Cholinesterase is associated with the basal lamina at the neuromuscular junction, *Nature* 271 (1978) 172-174.
- [25] Nachmansohn D., Changements de la cholinestérase dans le muscle strié, *C.R. Soc. Biol. (Paris)* 128 (1938) 599-603.
- [26] Nachmansohn D., Choline esterase in voluntary muscle, *J. Physiol. (Lond.)* 95 (1939) 29-35.

Allosteric nicotinic receptors, human pathologies

Clément Léna, Jean-Pierre Changeux*

CNRS UA 1284, Neurobiologie Moléculaire, Institut Pasteur, 25–28, rue du Dr-Roux, 75724 Paris cedex 15, France

Abstract — Nicotinic acetylcholine receptors are ligand-gated ion channels present in muscle and brain. These allosteric oligomers may exist in several conformational states which include a resting state, an open-channel state, and a desensitized refractory state. Recent work has shown that point mutations in the nicotinic receptor may, altogether, abolish desensitization, increase apparent affinity for agonists and convert the effect of a competitive antagonist into an agonist response. These pleiotropic effects are interpreted in terms of the allosteric model. This paper reviews recent evidence that such mutations occur spontaneously in humans and may cause diseases such as congenital myasthenia or familial frontal lobe epilepsy. In addition, nicotinic receptors are involved in tobacco smoking. Accumulating evidence, including experiments with knock-out animals, indicates that addiction to nicotine is linked to the activation of $\beta 2$ -subunit containing nicotinic receptors in the dopaminergic mesolimbic neurons which are part of the reward systems in the brain. Current research also indicates that nicotinic agonists might serve as therapeutic agents for Alzheimer's disease and Tourette's syndrome, as well as for schizophrenia. This paper extends and updates a recently published review. (©Elsevier, Paris)

Résumé — **Récepteurs nicotiniques allosteriques et pathologies humaines.** Les récepteurs nicotiniques sont des canaux activés par un neurotransmetteur présents dans le muscle et dans le cerveau. Ces oligomères allostériques peuvent exister dans différentes conformations telles qu'un état de repos, un état avec le canal ouvert et un état désensibilisé, réfractaire à l'activation. Des travaux récents ont montré que des mutations ponctuelles dans les récepteurs nicotiniques peuvent, d'un même coup, supprimer la désensibilisation, augmenter l'affinité apparente des agonistes et convertir l'effet des antagonistes compétitifs en réponse à un agoniste. Ces effets pléiotropiques sont interprétés à l'aide du modèle allostérique. Le présent article résume la découverte récente de l'occurrence spontanée de telles mutations chez l'humain et des pathologies qu'elles causent telles que des myasthénies congénitales et des épilepsies du lobe frontal. De plus, les récepteurs nicotiniques sont impliqués dans la tabagie. Un nombre croissant de travaux, notamment faisant usage d'animaux recombinants, démontre que la dépendance à la nicotine est liée à l'activation de récepteurs nicotiniques contenant la sous-unité $\beta 2$, dans les neurones dopaminergiques mésolimbiques qui participent aux systèmes de récompense dans le cerveau. Les travaux contemporains indiquent également que les agonistes nicotiniques pourraient remplir le rôle de médicament pour le traitement de la maladie d'Alzheimer, du syndrome de Gilles de la Tourette, et de la schizophrénie. Cet article est une mise à jour étendue d'une revue publiée antérieurement. (©Elsevier, Paris)

nicotinic acetylcholine receptors / allosteric proteins / congenital myasthenia / frontal lobe epilepsy / Alzheimer's disease / schizophrenia / nicotine addiction

1. Introduction

The nicotinic acetylcholine receptor (nAChR) was the first neurotransmitter receptor biochemically and functionally identified [2, 3], in part because high amounts of the receptor protein were available in the fish electric organ [4] and also because snake venom α -toxins had been identified as highly selective markers of nAChRs [5]. Recombinant DNA technologies further led to the demonstration that the structural and functional properties of this membrane allosteric protein from fish are, to a large extent, paralleled by those of brain nicotinic receptors thus opening the field to human brain pathologies [6, 7].

In this review, two aspects of nAChRs relevant to medicine are presented. First, point mutations in muscle and brain nAChRs may produce congenital myasthenia and familial epilepsies. The phenotypes

of the mutated nAChRs are interpreted in terms of changes in the properties of the allosteric transitions. Second, nicotinic drugs, despite their addictive properties, could potentially alleviate neurologic and psychiatric disorders.

2. Nicotinic receptors as allosteric membrane proteins

The nAChRs compose a family of ligand-gated ion channels differentially expressed in skeletal muscle and nerve cells (reviewed in [8–10]). They form 300 kDa transmembrane hetero- (or homo-) pentamers from a repertoire of 16 known different types of subunits referred to as $\alpha 1$ – $\alpha 9$, $\beta 1$ – $\beta 4$, γ , δ , and ϵ . The subunits are regularly distributed around an axis of quasi-symmetry delineating the ion channel (figure 1a, d). Each subunit contains a large N-terminal hydrophilic domain exposed to the synaptic cleft, followed by three transmembrane segments

* Correspondence and reprints.

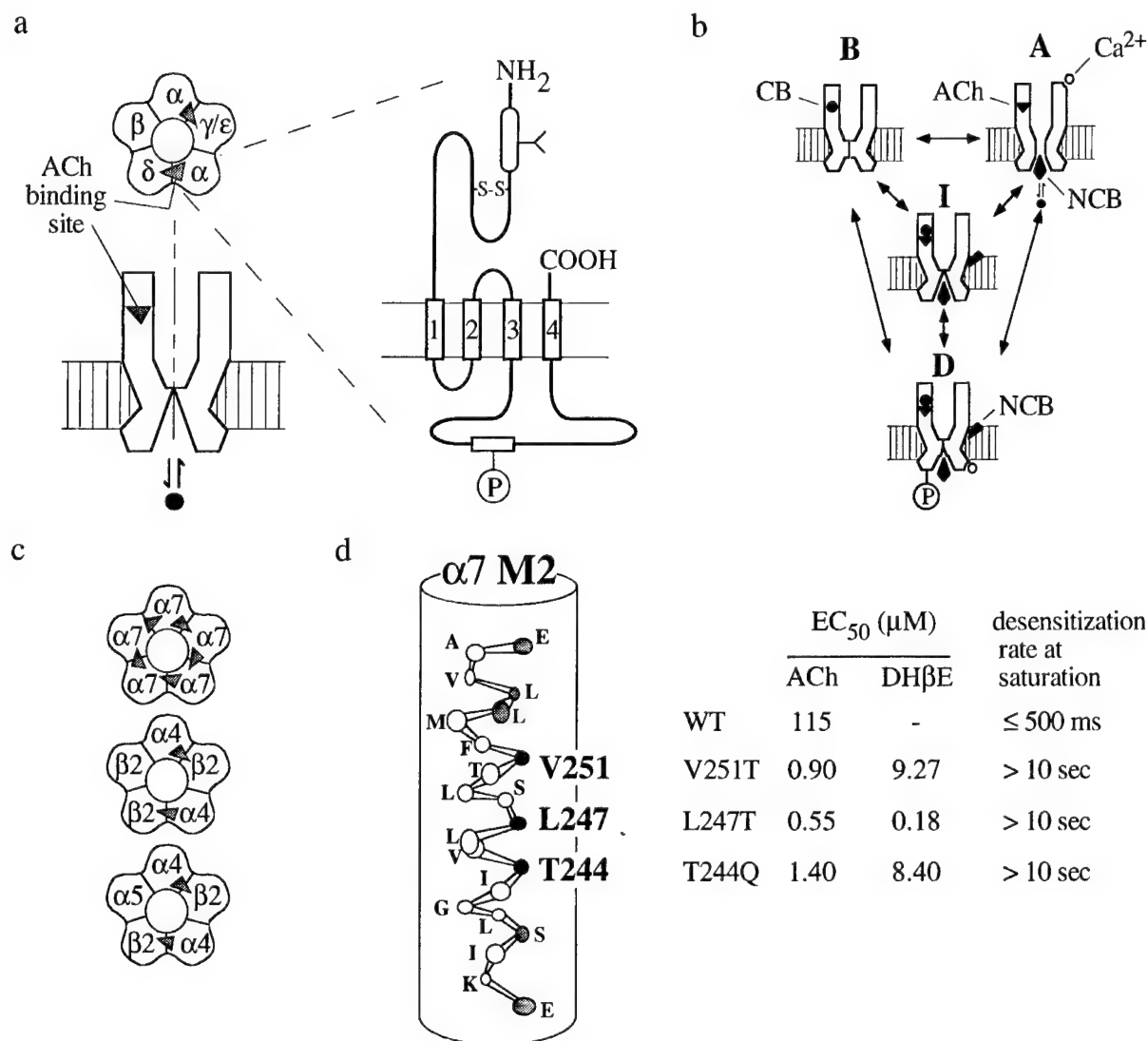


Figure 1. Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels with allosteric properties [30]. **a.** Muscle and *Torpedo* nAChRs are pentameric oligomers. The five homologous subunits are organized around an axis of quasi-symmetry perpendicular to plane of the plasma membrane, that delineates the ion channel pore. Each subunit exhibits a similar transmembrane organization sketched on the right. The binding sites are located at the interface of the extracellular N-terminal domains of the subunits, and the ion channel is lined by the M2 transmembrane segment. **b.** The nAChRs undergo allosteric transitions between a small number of states: resting (B), active (A) and desensitized (I, D) states [30, 31]. Various ligands preferentially bind to different states as indicated. CB, competitive blockers; NCB, non-competitive blockers; ACh, acetylcholine. **c.** Putative organization of three different types of neuronal nAChRs: homopentamers of $\alpha 7$ subunit, heteropentamers of $\alpha 4$ and $\beta 2$, heteropentamers of $\alpha 4$, $\beta 2$ and $\alpha 5$. **d.** The M2 transmembrane segment is putatively organized in an α -helix. Mutations of residues in M2 facing the channel pore increase the apparent affinity for nicotine, convert the antagonist dihydro- β -erythroidine into an agonist and drastically slow the desensitization rate of mutated $\alpha 7$ -nAChRs (adapted from [41]). Adapted from [1].

(M1–M3), a large intracellular loop and a C-terminal transmembrane segment (M4). Acetylcholine binding sites are located at the interface between α and non- α subunits in the N-terminal regions [8, 11–13]. They include a principal component of three loops

A, B, and C and a complementary component of at least three loops, D, E and F, on the non- α subunit. In homo-oligomeric receptors, the two components are carried by identical subunits [11, 14]. A wide diversity of binding properties in hetero-oligomers

results from the combinatorial diversity of the active site structure (e.g., [14]). The ion channel is lined by the M2 segment from each of the five subunits [15–21]. Neuronal nAChRs are more permeable to calcium ions than muscle nAChRs (neuronal nAChRs: pCa/pNa from 15 to 0.5 depending on the subunit composition; muscle nAChRs: pCa/pNa of about 0.2) [8, 9, 22].

Muscle nAChRs have a fixed composition $[\alpha 1]_2[\beta 1][\delta][\gamma \text{ or } \epsilon]$ in vertebrates. Neuronal nicotinic receptors are composed of neuron-specific subunits homologous to the muscle subunits. To date, ten neuronal subunits have been identified in mammals ($\alpha 2$ – $\alpha 7$, $\alpha 9$, $\beta 2$ – $\beta 4$) (e.g., [10, 23]). Of the more than 20 000 possible combinations of subunits, only a few yield functional receptors. The $\alpha 7$ and $\alpha 9$ subunits form functional homo-oligomers when expressed in *Xenopus* oocytes, while the $\alpha 2$ – $\alpha 4$, $\alpha 6$ subunits produce hetero-oligomers with the $\beta 2$ or the $\beta 4$ subunit (figure 1c, reviewed in [24, 25]). The $\alpha 5$ subunit can associate with $\alpha 3\beta 2/4$ and $\alpha 4\beta 2$ subunits and thus form hetero-oligomers with three different subunits (figure 1c; [26, 27]). The sequence homology of $\beta 3$ with the $\alpha 5$ subunit suggests that $\beta 3$ possesses a similar function [28], and is also integrated into functional nAChRs [29].

Upon application of nicotinic agonists, both muscle and neuronal nAChRs undergo fast activation leading to an open-channel state, and a slow desensitization reaction leading to a closed-channel state refractory to activation. Activation and desensitization of muscle and brain nAChRs correspond to transitions between a small number of discrete structural states with distinct binding properties and ion channel conductance [30]. Consistent with the allosteric MWC model and its extension to membrane receptors [30, 31], the different conformational states may spontaneously exist in the absence of ligands, and nicotinic effectors cooperatively modify the equilibrium and kinetic constants for the transitions between the states (figure 1b). The pharmacological and kinetic characteristics of these states depend upon the subunit composition. Indeed, the two main subtypes of brain nAChRs strikingly differ: the human $\alpha 4\beta 2$ and $\alpha 7$ have respectively a low and a high EC_{50} for nicotine (0.3–5 μ M versus 40–110 μ M); at saturation, they desensitize respectively in the 10-s and in the 10–100 ms range (or less) [32–36]. The kinetic constants governing the ligand binding and the transitions between the different states (14 independent rate constants for a four state model) have been estimated for muscle nAChR [31] and the analysis extended to neuronal nAChR mutants [31, 37, 38].

Site-directed mutagenesis of affinity-labeled residues in the channel and active site domains [39–43]

revealed that mutations of single amino acids can modify multiple functions of the nAChR. For instance, mutations in the channel lining region M2 (e.g., $\alpha 7T244Q$, $\alpha 7L247T$, $\alpha 7V251T$) produce a 100-fold increase in apparent affinity for agonists, a loss of desensitization and a conversion of competitive antagonists to agonists (figure 1d) [39, 41–43] (reviewed in [8, 37]). The allosteric model accounts for these pleiotropic phenotypes. Different classes of phenotypes may be associated with selective changes either in the binding properties (K phenotype), in the biological activity of the ion channel (γ -phenotype) or in the isomerization constants between receptor conformations (L-phenotype) and possibly in both [37, 44].

The neuronal nicotinic receptor subunits are expressed differentially in the mammalian brain. In situ hybridization in the rat brain shows that $\alpha 4$, $\beta 2$ and $\alpha 7$ are widely expressed, that $\alpha 3$ and $\alpha 5$ are less ubiquitous and that $\alpha 6$, $\beta 3$, $\beta 4$ and $\alpha 2$ are only expressed in a few brain structures (table 1). In contrast, $\alpha 3$ and $\beta 4$ are the most abundant nAChR subunits in the autonomic peripheral nervous system [45]. The distribution of nAChRs has also been studied with radiolabeled nicotinic ligands. At least four different types of nicotinic-ligand binding activity with different distributions have been distinguished: the high affinity binding sites for nicotine that correspond to $\beta 2$ -containing nAChRs [46, 47] are found throughout the brain (e.g., [48]) and are also labeled by three other nicotinic ligands: acetylcholine (in the presence of atropine to block muscarinic ligands), cytosine and epibatidine. The high-affinity binding sites for epibatidine (but not for nicotine) have a much restricted localization limited mostly to the habenulo-interpeduncular system and the dorsal medulla oblongata; these sites may be separated into two families, since only a subset of them binds cytosine with a high affinity [47] (see also [49]). They correspond presumably to $\beta 4$ -containing nAChRs [4]. The α -bungarotoxin binding sites that correspond to $\alpha 7$ -containing nAChRs [50] are distributed throughout the brain with a prevalent localization in the limbic system. As a consequence of such diversity in function and distribution, nAChRs may contribute to a wide array of brain functions [51]. Conversely, dysfunction of a single nAChR subunit may produce diverse deficits.

3. Congenital myasthenia and familial epilepsies result from nAChR point mutation

Genetic analysis of several human (and animal) pathologies has revealed nAChR mutations yielding pleiotropic phenotypes (figure 2). The mutations are

Table I. Differential distribution of nAChR subunit mRNA in the rat brain ([28] and references therein). Recent work suggests a broader expression pattern of the subunits $\alpha 3$ and $\beta 4$ observed by in situ hybridization [112]. However such observations do not seem to be consistent with recent binding experiments in $\beta 2$ -knockout mice [47]. The expression of $\alpha 7$ in the dopaminergic nuclei (DA nuclei) was recently demonstrated [91]. NTS, nucleus of the tractus solitarius. Adapted from [1].

	$\alpha 2$	$\alpha 3$	$\alpha 4$	$\alpha 5$	$\alpha 6$	$\alpha 7$	$\beta 2$	$\beta 3$	$\beta 4$
Telencephalon									
olfactory bulb	+	++	+	++	-	++	++	-	+
isocortex									
layer II-III	-	-	+	+	-	+	++	-	-
layer IV	-	+	+	+	-	+	++	-	-
layer V	-	-	++	+	-	++	++	-	-
layer VI	-	-	++	++	-	++	++	-	-
hippocampal formation	(+)	(+)	+	+	-	+++	++	-	-
striatum	-	-	-	-	-	-	+	-	-
septum	-	-	+	-	-	+	+	-	-
hypothalamus	-	-	+	-	-	-	+	-	-
supraoptic n.	-	-	+	-	-	+++	+	-	-
Diencephalon									
pineal gland	-	+++	-	+	-	-	+	-	+++
habenula	-	+++	++	+	(+)	(+)	++	++	+++
thalamus	-	+	+++	-	+	-	+++	+	-
Mesencephalon									
DA nuclei	-	(+)	++	++	+++	(+)	++	+++	-
mesenc V nucleus	-	-	+	-	+	-	++	+++	-
interpeduncular nucleus	++	+	+	++	+	(+)	++	+	+
Rhombencephalon									
vestibular nuclei	-	-	+	+	-	++	+	-	-
cerebellum	-	+	-	+	-	-	+	+	+
locus coeruleus	-	(+)	-	-	+++	-	++	+++	(+)
motor nuclei	-	+	+	+	-	-	++	-	-
NTS	-	++	(+)	+	-	-	++	-	++
area postrema	-	++	++	+	-	-	++	-	+

homologous or even identical to those initially studied in reconstituted $\alpha 7$ homo-oligomers and their phenotype may be also interpreted in terms of the allosteric model.

A mutation in the *deg-3* gene coding for a putative nAChR subunit of the nematode *C. elegans* results in neurodegeneration [52]. This *deg3-I293N* mutation is likely to cause an 'increase-of-function' similar to that initially found with the vertebrate $\alpha 7V251T$ mutation [41]. The neurotoxicity could plausibly arise from a large toxic influx of calcium associated with a non-desensitizing and/or spontaneously open nAChR channel [39, 41].

In humans, myasthenia gravis is a sporadic disease caused by an auto-immune reaction directed against muscle nAChRs. However, some congenital myasthenic syndromes are associated with point mutations in the muscle $\alpha 1$, $\beta 1$ or ϵ subunits. Mutations reducing channel opening transitions (e.g., $\epsilon P121L$,

$\epsilon I254ins18$), or affecting nAChR assembly ($\epsilon R147L$) cause myasthenic symptoms only when combined with a null mutation of the other allele [53–55]. Null mutations [56] result in symptoms only when expressed on both alleles. In accordance, animal models with a knock-out of the subunit ϵ express obvious myasthenic symptoms only in a homozygous genotype [57]. In these myasthenic patients and animal models, neurotransmission is partially rescued at the neuromuscular junction by the persistence of expression of the fetal nAChR γ subunit.

Mutations increasing the time spent by nAChRs in the open state also produce myasthenic syndromes, even as heterozygous mutations [58–63] (but see [54]). Mutations causing such 'increase-of-function' phenotypes occur near the ligand binding region ($\alpha 1G153S$, $\alpha 1V156M$), in the transmembrane M2 segment delineating the ion channel ($\alpha 1T254I$,

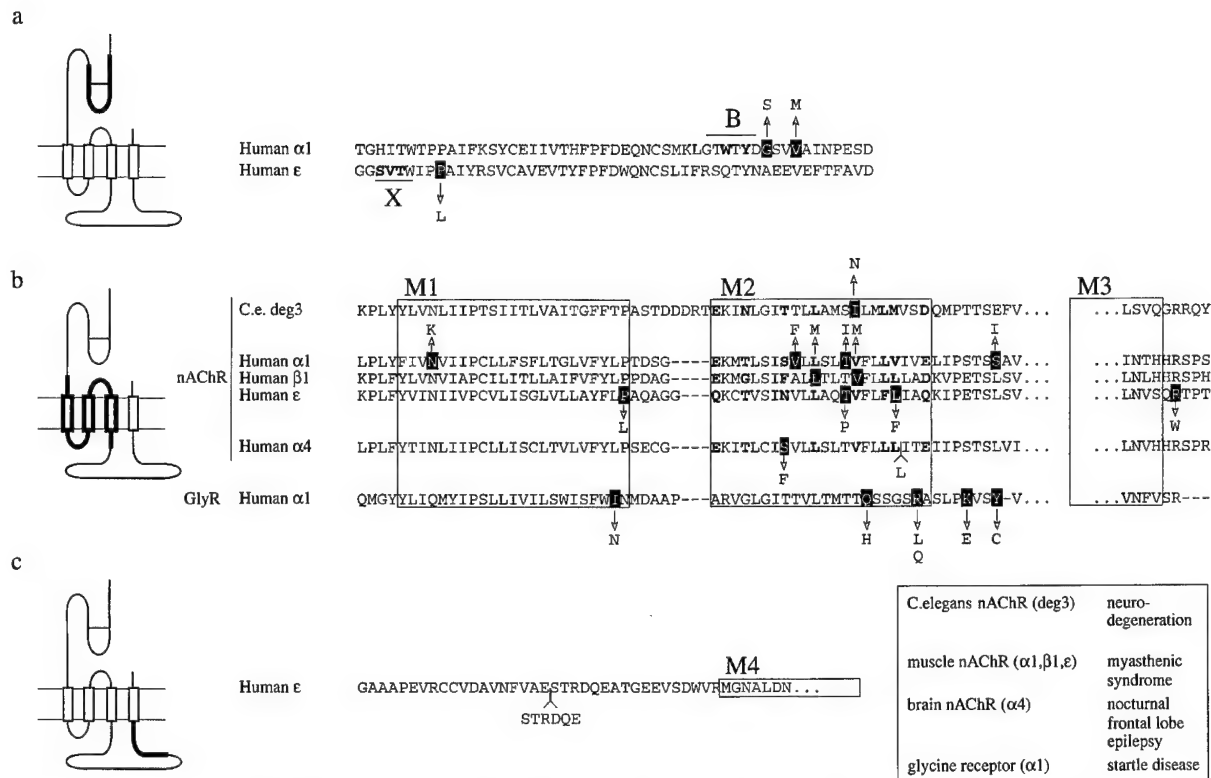


Figure 2. Pathogenic mutations affecting allosteric transitions in the muscle and neuronal nicotinic receptors and in the homologous glycine receptor. Each disease is caused by a single mutation among the mutations indicated. The drawings on the left indicate the protein domain concerned. The mutations are indicated by the amino acid symbol above/under the sequence with an arrow pointing from the wild-type highlighted residue. Null mutations are not presented here. **a.** Mutations near the ligand binding regions. B, B-loop in the principal component of the binding site. X, unnamed region in the complementary component of the binding site [12]. The mutations drawn are: ϵ P121L [53], $\alpha 1$ G153S [59, 62] and $\alpha 1$ V156M [62]. **b.** Mutations in/near the transmembrane domains. Note the large number of mutations in the M2 region. The amino acids facing the channel pore are indicated in bold. The mutations in nAChR subunit gene are *deg3*1293N [52], $\alpha 1$ N217K [60], $\alpha 1$ V249F [63], $\alpha 1$ T254I [62], $\alpha 1$ S269I [62], $\beta 1$ L262M [61], $\beta 1$ V266M [60], ϵ P245L [54], ϵ T264P [58], ϵ L269F [60, 111], ϵ R311W [54], $\alpha 4$ S248F [66], $\alpha 4$ -776 (ins3) [69]. The mutations in the $\alpha 1$ glycine receptor gene are: GlyR $\alpha 1$ -I244N, GlyR $\alpha 1$ -Q266H, GlyR $\alpha 1$ -R271Q, GlyR $\alpha 1$ -R271L, GlyR $\alpha 1$ -K276E, GlyR $\alpha 1$ -Y279C (references in [70]). Alignment was performed with the program Clustalw of DG Higgins and PM Sharp. **c.** The mutation in the intracellular loop between the M3 and M4 segments is ϵ L254ins18 [55]. Adapted from [1].

$\alpha 1$ V249F, $\beta 1$ V266M, $\beta 1$ L262M, ϵ L269F) or in adjacent regions ($\alpha 1$ N217K, $\alpha 1$ S269I, ϵ P245L) (figure 2). They may affect both intrinsic ligand binding (K phenotype) and opening transition/desensitization (L phenotype) processes or both [37, 38, 44]. Neighboring mutations may produce different phenotypes; for instance, $\alpha 1$ G153S slows agonist dissociation while $\alpha 1$ V156M decreases the rate of channel closing [59, 62]. Some mutations ($\alpha 1$ V249F, $\beta 1$ V266M, ϵ T264P, ϵ L269F) produce a high rate of spontaneous openings in the absence of ligand [58, 60, 63], a phenotype consistent with the allosteric model on the

basis of a shift of the allosteric equilibrium in favor of the open state [37, 38, 64]. The confirmation that such 'increase-of-function' mutations in muscle nAChRs are pathogenic was recently obtained in an animal model of transgenic mice carrying the ϵ L269F mutation [65].

Recently, some familial epilepsies have been linked to mutations in the $\alpha 4$ nAChR subunit. In an Australian family, an $\alpha 4$ S248F mutation was found to produce autosomal dominant frontal lobe epilepsy [66]. The mutated serine faces the channel pore, as initially demonstrated by chlorpromazine labeling in

Torpedo nAChRs (references in [8]). The mutation of the homologous residue in brain $\alpha 7$ nAChRs ($\alpha 7$ T244, *figure 1d*) causes drastic changes in the affinity for ACh and the desensitization properties of the nAChRs [41]. In the human $\alpha 4$ gene, $\alpha 4$ S248F produces a limited change in the apparent affinity for acetylcholine and a five-fold increase in the desensitization rate of $\alpha 4\beta 2$ nAChRs [67], a decrease in the mean single channel conductance and a loss of calcium permeability [68]. Furthermore, this mutant exhibited an 'use-dependent potentiation' of the electrophysiological response to nicotinic agonists [68]. In a Norwegian family, the same epileptic syndrome was linked to the insertion of a GCT triplet at nucleotide 776, resulting in the insertion of a leucine at codon 260 [69]. In oocyte experiments, this insertion causes a 12-fold increase in the apparent affinity for ACh of human $\alpha 4\beta 2$. As the insertion is adjacent to a pair of leucines previously identified as a critical element for calcium permeability [22], it might also reduce calcium permeability, though definitive evidence is lacking for this mutant. It is unclear for both mutations $\alpha 4$ S248F, $\alpha 4$ (776ins3) whether the phenotype is due to a 'loss of function' (increased desensitization rate and loss of calcium permeability) or to an 'increase of function' (increase in apparent affinity, 'use-dependent' potentiation).

Point mutations that change allosteric properties occur in other ligand-gated ion channels, such as the glycine receptor $\alpha 1$ subunit. Human hereditary hyperekplexia is caused by mutations in the M1-M2 and M2-M3 loops that lead to a dramatic reduction of efficacy of the agonist ([70] and references therein). Overall, these results show that point mutations can cause either a loss of function or an apparent 'increase of function' by altering the allosteric transitions of the nAChRs. Increase-of-function mutations occur frequently and may be as pathogenic as null mutations. Since each allosteric state of the nAChR possesses a distinct pharmacological profile, one may anticipate the development of novel pharmacological agents targeted not only to a particular combination of subunits but to each of the diverse conformations spontaneously accessible by the various receptor oligomers.

4. Null mutation of neuronal nAChRs, Alzheimer's disease and memory

The role of defined nAChR subunits in brain function has been examined using knock-out mice. Mice lacking the most widely expressed $\beta 2$ subunit survive, feed and mate normally [46]. Their brains are of a normal size and morphology. The high affinity

nicotine binding sites (classically attributed to $\alpha 4\beta 2$ nAChRs) completely disappear from the brain of homozygous mutant mice, whereas the α -bungarotoxin sites (corresponding to the $\alpha 7$ -containing nAChRs) persist. Electrophysiological responses to nicotine are no longer recorded in the thalamus but persist in a few structures expressing the $\beta 4$ nAChR subunit (such as the medial habenula) [46, 47]. Further analysis of the $\beta 2$ mutant mice has shown that the $\beta 2$ -containing nAChRs are expressed both in the somato-dendritic compartment, and in the axonal compartment of neurons as presynaptic nAChRs [71]. The absence of the $\beta 2$ subunit affects the performance in associative memory (passive avoidance) tests of mutant animals and suppresses the improvement of the performance by nicotine [46]. Activation of $\beta 2$ -containing nAChRs by endogenous ACh is thus likely to take place in the course of these memory tasks.

Preliminary results indicate that the knock-out of the $\alpha 7$ subunit yields animals that survive normally but display an anomalous synchronisation during EEG recordings [72].

Nicotine enhancement of memory processes has motivated clinical trials of nicotinic treatment in the Alzheimer's disease (AD). The severity of symptoms in AD is well correlated with a reduction in cortical acetylcholine ([73] and references therein) and AD patients exhibit a marked reduction in the number of high affinity nicotine binding sites [74]. Nicotine treatment partially relieves the cognitive deficits of AD [75, 76]. The site of this beneficial action of nicotine is not yet clearly established. For instance, nicotine may increase the levels of ACh in the cortex by recruiting presynaptic nAChRs on ACh terminals in the cortex (e.g., [77]). As the use of nicotine presents a number of side effects linked to the activation of peripheral nAChRs, attempts are made to find nicotinic drugs specific for brain subtypes such as ABT418 [78], SIB-1508Y [79] and RJR 2403 [80].

5. Nicotinic receptors in the reward system and tobacco abuse

The nAChRs subunits are abundantly expressed in the mesencephalic dopaminergic nuclei ([28] and references therein). These nuclei are part of the mesostriatal reward system. Theoretical work has underlined the critical function of reward systems in learning of behavioral rules by selection [81, 82]. Dysfunction or anomalous chemical stimulation of these systems strongly affects brain function. Indeed, the mesostriatal dopaminergic system is a common target of many addictive drugs (reviewed in [51, 83]).

Accumulating data suggest that both tobacco smoking in humans and nicotine self-administration in animals are associated with an increase in dopamine release following nicotinic actions on the mesencephalic dopaminergic neurons (reviewed in [84]). Self-administration of nicotine shares common mechanisms with that of other addictive drugs. Minimal doses of nicotine, comparable to those causing self-administration behavior, trigger a specific increase of metabolism and the release of dopamine in the nucleus accumbens, as observed with strongly addictive drugs such as cocaine or amphetamines [85]. Nicotine and cocaine self-administration activates a number of common brain structures, as visualized with c-Fos immunoreactivity, notably the terminal fields of the mesencephalic dopaminergic neurons [86]. It should be noted that tobacco smoking is not only associated with nicotine intake but also with respiratory sensations of smoke intake (review in [84]); nicotine action could also be amplified by changes in dopamine metabolism, since smokers display a 40% reduction of monoamine oxidase B compared to former smokers or non-smokers [87].

What is the composition of the nAChRs involved in the self-administration of nicotine? The concentration of nicotine in the plasma of smokers is in the 100–500 nM range [88]. Oocyte experiments with human nAChRs reveal which combinations of subunits may respond to such low concentration of agonists in vivo. EC₅₀ values below 10 μ M have been observed for $\alpha 4\beta 2$, $\alpha 4\beta 4 \alpha 3\beta 2$ ([33] but see [36]) and $\alpha 3\beta 2\alpha 5$ [27]. In situ hybridization experiments (references in [28]) indicate that the nAChR subunits forming these combinations (except $\beta 4$) are expressed in the mesencephalic nuclei. These nuclei also contain high amounts of the $\alpha 6$ and $\beta 3$ subunit mRNAs, suggesting the contribution of an $\alpha 6\beta 3\beta 2$ subtype [28]. Recent work on the $\beta 2$ -knock-out mice show that the $\beta 2$ -mutant animals no longer self-administer nicotine after priming by cocaine, indicating that $\beta 2$ is part of the nAChRs involved in nicotine reinforcement [89]. Furthermore, in the mutant animals low concentrations of nicotine fail to stimulate the mesencephalic dopaminergic neurons recorded on slices in vitro and systemic nicotine injection no longer enhances the striatal release of dopamine [89].

The chronic intake of nicotine results in a so-called 'neuronal adaptation'. This includes desensitization of nAChRs (reviewed in [90]) [91]. Desensitization of neuronal nicotinic receptors may recover only in tens of minutes (e.g., [91–94]) and thus likely contributes to short term adaptation to nicotine. Furthermore, nAChRs may directly contribute to endogenous reward processes. Indeed, they contribute to cholinergic synaptic transmission be-

tween the posterior cholinergic nuclei (latero-dorsal tegmental nucleus, pedunculopontine nucleus) and the mesencephalic dopaminergic neurons (e.g., [95, 96]). Chronic nicotine treatment thus likely affects the brain reward mechanisms as shown with self-stimulation protocols [97] or nicotine withdrawal experiments [98].

6. The nicotinic receptors in psychiatric and neurological disorders

The high prevalence of tobacco-smoking in schizophrenic patients suggests that nicotine intake by cigarette consumption may be a form of self-medication. By stimulating the mesencephalic dopaminergic system (see above), and more specifically by increasing the burst firing of dopaminergic neurons to burst activity, nicotine might compensate for the hypofrontality observed in schizophrenia [9]. Nicotine has been found to reverse the cognitive deficits produced by haloperidol in schizophrenics [100]. A synergy between nicotine and dopaminergic neuroleptics also exists in the treatment of Tourette's syndrome [101]. Nicotine has been proposed as an alternative to drugs increasing the brain levels of dopamine in the treatment of attention deficit/hyperactivity disorder [102]. Finally, nicotine and various nicotinic agonists might help to compensate the deficit in striatal dopamine in Parkinson's disease patients and might, in some instances, relieve the symptom of the disease ([103] but see [104]). The interaction of nicotine with the mesencephalic dopaminergic system might thus explain its action on psychiatric and neurological disorders.

Schizophrenic patients often exhibit, among diverse symptoms, a diminished habituation to auditory stimulation (reviewed in [105]). Experiments in the rodent have shown that auditory gating is impaired by antagonists of $\alpha 7$ nAChRs. Furthermore, the number of α -bungarotoxin sites is reduced in post-mortem brains of schizophrenics. The deficit of sensory gating in schizophrenics might thus be due to a reduction, or a loss, of $\alpha 7$ nAChR function. Consistent with this hypothesis, genetic analysis in nuclear families with at least two cases of schizophrenia has shown that the deficit in auditory gating is significantly linked to a genetic marker neighboring the locus of the $\alpha 7$ gene [106]. A study of the relatives of schizophrenics sharing the deficit in auditory gating revealed that nicotine could reverse the deficit, presumably by activating $\alpha 7$ nAChRs (references in [105]). This result is quite unexpected, since human $\alpha 7$ nAChRs exhibit a low sensitivity to nicotine (see *Introduction*). However, recent experiments in the chick [107] and rat [108] have

shown that low doses of nicotine can activate α -bungarotoxin sensitive nAChRs in glutamatergic nerve terminals. The $\alpha 7$ nAChR subunit, possibly together with still unidentified subunit(s) (see however, [109]), may thus form another relevant target for nicotinic therapies of psychiatric disorders.

7. Conclusion

Over 25 years after the identification and purification of the *Torpedo* nAChR (see [30]), the accumulating knowledge on the nAChRs in vertebrates has led to the demonstration that alterations of these receptors are responsible for a variety of familial disorders of the central and peripheral nervous system. Conversely, these receptors are now considered as relevant targets for nicotinic therapies of brain disorders.

Previous experiments combining photoaffinity labeling and site-directed mutagenesis had shown that changes of critical amino acids, in the nAChR channel or ligand binding site, may markedly affect its function in a pleiotropic manner. They may for instance, either reduce or increase channel opening in the presence and sometimes in the absence of acetylcholine by altering the allosteric properties of the protein. Interestingly, analogous, if not identical, point mutations in human nAChR genes (and glycine receptor genes) have been shown to cause pathologies either by a loss or by an 'increase-of-function'. Mutations causing pathologies via changes in allosteric properties have also been described for G-protein linked receptors [110]. Development of novel nicotinic therapies with pharmacological agents targeted to these diverse 'allosteric' phenotypes may thus be anticipated.

While the strategic location of nAChRs in the dopaminergic reward system renders nicotine an addictive drug, it also underlies potential beneficial effects of nicotine in the treatment of psychiatric disorders. Furthermore, nAChRs may relieve symptoms of AD or schizophrenia via pathways different from the dopaminergic system; nicotinic agents which specifically activate nAChR subtypes absent from the dopaminergic system, and thus with no (or diminished) addictive properties should, therefore, be looked for.

Fundamental research on properties of nAChRs in normal and pathological situations opens many new strategies to design drug therapies targeted not only to specific nAChRs in defined brain circuits but also to specific allosteric transitions impaired by nAChR gene mutations in humans.

Acknowledgments

We thank A. Devillers-Thiery, S. Edelstein, N. Le Novère, R. Miles, M. Picciotto and M. Zoli for critical reading of the manuscript, Y. Paas and N. Le Novère for their help with the table and figures. This work was supported by grants from the Collège de France, the Centre National de la Recherche Scientifique, the Association Française contre les Myopathies, Direction des Recherches et Etudes Techniques, the Council for Tobacco Research and the EEC Biotech and Biomed Programs. C.L. is supported by the Association France Alzheimer.

References

- [1] Léna C., Changeux J.P., Pathological mutations of nicotinic receptors and nicotine-based therapies for brain disorders, *Curr. Opin. Neurobiol.* 7 (1997) 674–682.
- [2] Changeux J.P., Kasai M., Lee C.Y., The use of a snake venom toxin to characterize the cholinergic receptor protein, *Proc. Natl. Acad. Sci. USA* 67 (1970) 1241–1247.
- [3] Changeux J.P., The acetylcholine receptor: an 'allosteric' membrane protein, in: *Harvey Lectures*, Academic Press Inc., 1981, pp. 85–254.
- [4] Nachmansohn D., *Chemical and molecular basis of nerve activity*, Academic Press New York, 1959.
- [5] Lee C.Y., Chang C.C., Modes of actions of purified toxins from elapid venoms on neuromuscular transmission, *Mem. Inst. Butantan Sao Paulo*, 33 (1966) 555–572.
- [6] Numa S., A molecular view of neurotransmitter receptors and ionic channels, *Harvey Lecture Series* 83 (1989) 121–165.
- [7] Heinemann S., Boulter J., Deneris E., Connolly J., Gardner P., Wada E., Wada K., Duvoisin R., Ballivet M., Swanson L., Patrick J., Brain and muscle nicotinic acetylcholine receptor: a gene family., in: Maelicke A. (Ed.), *Molecular Biology of Neuroreceptors and Ion Channels*, Springer-Verlag, Berlin Heidelberg, 1989, pp. 13–30.
- [8] Galzi J.L., Changeux J.P., Neuronal nicotinic receptors: molecular organization and regulations, *Neuropharmacology* 34 (1995) 563–582.
- [9] Role L.W., Berg D.K., Nicotinic receptors in the development and modulation of CNS synapses, *Neuron* 16 (1996) 1077–1085.
- [10] Le Novère N., Changeux J.P., Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells, *J. Mol. Evol.* 40 (1995) 155–172.
- [11] Corringer P.J., Galzi J.L., Eiselé J.L., Bertrand S., Changeux J.P., Bertrand D., Identification of a new component of the agonist binding site of the nicotinic $\alpha 7$ homooligomeric receptor, *J. Biol. Chem.* 270 (1995) 11749–11752.
- [12] Tsigelny I., Sugiyama N., Sine S.M., Taylor P., A model of the nicotinic receptor extracellular domain based on sequence identity and residue location, *Biophys. J.* 73 (1997) 52–66.
- [13] Karlin A., Akabas M.H., Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins, *Neuron* 15 (1995) 1231–1244.
- [14] Corringer P.J., Bertrand S., Bohler S., Edelstein S.J., Changeux J.P., Critical elements determining diversity in agonist binding and desensitization of neuronal nicotinic acetylcholine receptors, *J. Neurosci.* 18 (1998) 648–657.

- [15] Giraudat J., Dennis M., Heidmann T., Chang J.Y., Changeux J.P., Structure of the high affinity site for noncompetitive blockers of the acetylcholine receptor: serine-262 of the delta subunit is labeled by [³H]-chlorpromazine, *Proc. Natl. Acad. Sci. USA* 83 (1986) 2719-2723.
- [16] Giraudat J., Dennis M., Heidmann T., Haumont P.Y., Lederer F., Changeux J.P., Structure of the high-affinity binding site for noncompetitive blockers of the acetylcholine receptor: [³H] chlorpromazine labels homologous residues in the beta and delta chains. *Biochemistry* 26 (1987) 2410-2418.
- [17] Hucho F., Oberthür W., Lottspeich F., The ion channel of the nicotinic acetylcholine receptor is formed by the homologous helices M2 of the receptor subunits, *FEBS Lett.* 205 (1986) 137-142.
- [18] Leonard R.J., Labarca C.G., Charnet P., Davidson N., Lester H.A., Evidence that the M2 membrane-spanning region lines the ion channel pore of the nicotinic receptor, *Science* 242 (1988) 1578-1581.
- [19] Imoto K., Methfessel C., Sakmann B., Mishina M., Mori Y., Konno T., Fukuda K., Kurasaki M., Bujo H., Fujita Y., Numa S., Location of a delta-subunit region determining ion transport through the acetylcholine receptor channel. *Nature* 324 (1986) 670-674.
- [20] Imoto K., Busch C., Sakmann B., Mishina M., Konno T., Nakai J., Bujo H., Mori Y., Fukuda K., Numa S., Rings of negatively charged amino acids determine the acetylcholine receptor channel conductance, *Nature* 335 (1988) 645-648.
- [21] Cohen J.B., Blanton M.P., Chiara D.C., Sharp S.D., White B.H., Structural organization of functional domains of the nicotinic acetylcholine receptor, *J. Biol. Chem.* 3 (1992) 217.
- [22] Bertrand D., Galzi J.L., Devillers-Thiery A., Bertrand S., Changeux J.P., Mutations at two distinct sites within the channel domain M2 alter calcium permeability of neuronal alpha7 nicotinic receptor, *Proc. Natl. Acad. Sci. USA* 90 (1993) 6971-6975.
- [23] Gotti C., Fornasari D., Clementi F., Human neuronal nicotinic receptors, *Prog. Neurobiol.* 53 (1997) 199-237.
- [24] McGehee D.S., Role L.W., Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons, *Annu. Rev. Physiol.* 57 (1995) 521-546.
- [25] Fucile S., Matter J.M., Erkman L., Ragozzino D., Barabino B., Grassi F., Alema S., Ballivet M., Eusebi F., The neuronal alpha6 subunit forms functional heteromeric acetylcholine receptors in human transfected cells, *Eur. J. Neurosci.* 10 (1998) 172-178.
- [26] Ramirez Latorre J., Yu C.R., Qu X., Perin F., Karlin A., Role L., Functional contributions of alpha5 subunit to neuronal acetylcholine receptor channels, *Nature* 380 (1996) 347-351.
- [27] Wang F., Gerzanich V., Wells G.B., Anand R., Peng X., Keyser K., Lindstrom J., Assembly of human neuronal nicotinic receptor alpha5 subunits with alpha3, beta2, and beta4 subunits, *J. Biol. Chem.* 271 (1996) 17656-17665.
- [28] Le Novère N., Zoli M., Changeux J.P., Neuronal nicotinic receptor alpha6 subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain, *Eur. J. Neurosci.* 8 (1996) 2428-2449.
- [29] Rogers M., Colquhoun L.M., Patrick J.W., Dani J., Calcium flux through predominantly independent purinergic ATP and nicotinic acetylcholine receptors, *J. Neurophysiol.* 77 (1997) 1407-1417.
- [30] Changeux J.P., Functional architecture and dynamics of the nicotinic acetylcholine receptor: an allosteric ligand-gated ion channel, in: Changeux J.P. et al. (Eds.), *Fidia Research Foundation Neuroscience Award Lectures*, Raven Press Ltd., New York. 1990, pp. 21-168.
- [31] Edelstein S.J., Schaad O., Henry E., Bertrand D., Changeux J.P., A kinetic mechanism for nicotinic acetylcholine receptors based on multiple allosteric transitions, *Biol. Cybern.* 75 (1996) 361-379.
- [32] Peng X., Katz M., Gerzanich V., Anand R., Lindstrom J., Human alpha7 acetylcholine receptor: cloning of the alpha7 subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional alpha7 homomers expressed in *Xenopus* oocytes, *Mol. Pharmacol.* 45 (1994) 546-554.
- [33] Gerzanich V., Peng X., Wang F., Wells G., Anand R., Fletcher S., Lindstrom J., Comparative pharmacology of epibatidine: a potent agonist for neuronal nicotinic acetylcholine receptors, *Mol. Pharmacol.* 48 (1995) 774-782.
- [34] Gopalakrishnan M., Buisson B., Touma E., Giordano T., Campbell J.E., Hu I.C., Donnelly R.D., Arneric S.P., Bertrand D., Sullivan J.P., Stable expression and pharmacological properties of the human alpha7 nicotinic acetylcholine receptor, *Eur. J. Pharmacol.* 290 (1995) 237-246.
- [35] Buisson B., Gopalakrishnan M., Arneric S.P., Sullivan J.P., Bertrand D., Human alpha4beta2 neuronal nicotinic acetylcholine receptor in HEK 293 cells: A patch-clamp study, *J. Neurosci.* 16 (1996) 7880-7891.
- [36] Chavez Noriega L.E., Crona J.H., Washburn M.S., Urrutia A., Elliott K.J., Johnson E.C., Pharmacological characterization of recombinant human neuronal nicotinic acetylcholine receptors h-alpha2beta2, h-alpha2beta4, h-alpha3beta2, h-alpha3beta4, h-alpha4beta2, h-alpha beta4 and h-alpha7 expressed in *Xenopus* oocytes, *J. Pharmacol. Exp. Ther.* 280 (1997) 346-356.
- [37] Edelstein S.J., Schaad O., Changeux J.P., Myasthenic nicotinic receptor mutant interpreted in terms of the allosteric model, *C. R. Acad. Sci. Paris* 320 (1997) 953-961.
- [38] Edelstein S., Schaad O., Changeux J.P., Single binding versus single channel recordings: a new approach to study ionotropic receptors, *Biochemistry* 36 (1997) 13755-13760.
- [39] Revah F., Bertrand D., Galzi J.L., Devillers-Thiery A., Mulle C., Hussy N., Bertrand S., Ballivet M., Changeux J.P., Mutations in the channel domain alter desensitization of a neuronal nicotinic receptor, *Nature* 353 (1991) 846-849.
- [40] Galzi J.L., Bertrand D., Devillers-Thiery A., Revah F., Bertrand S., Changeux J.P., Functional significance of aromatic amino acids from three peptide loops of the alpha7 neuronal nicotinic receptor site investigated by site-directed mutagenesis, *FEBS Lett.* 294 (1991) 198-202.
- [41] Devillers-Thiery A., Galzi J.L., Bertrand S., Changeux J.P., Bertrand D., Stratified organization of the nicotinic acetylcholine receptor channel, *Neuroreport* 3 (1992) 1001-1004.
- [42] Bertrand D., Devillers-Thiery A., Revah F., Galzi J.L., Hussy N., Mulle C., Bertrand S., Ballivet M., Changeux J.P., Unconventional pharmacology of a neuronal nicotinic receptor mutated in the channel domain, *Proc. Natl. Acad. Sci. USA* 89 (1992) 1261-1265.
- [43] Labarca C., Nowak M.W., Zhang H., Tang L., Deshpande P., Lester H.A., Channel gating governed symmetrically by conserved leucine residues in the M2 domain of nicotinic receptors, *Nature* 376 (1995) 514-516.
- [44] Galzi J.L., Edelstein S.J., Changeux J., The multiple phenotypes of allosteric receptor mutants, *Proc. Natl. Acad. Sci. USA* 93 (1996) 1853-1858.
- [45] Zoli M., Le Novère N., Hill J.J., Changeux J.P., Developmental regulation of nicotinic ACh receptor subunit

- mRNAs in the rat central and peripheral nervous systems, *J. Neurosci.* 15 (1995) 1912–1939.
- [46] Picciotto M.R., Zoli M., Léna C., Bessis A., Lallemant Y., Le Novère N., Vincent P., Merlo Pich E., Brûlet P., Changeux J.P., Abnormal avoidance learning in mice lacking functional high-affinity nicotine receptor in the brain, *Nature* 374 (1995) 65–67.
- [47] Zoli M., Léna C., Picciotto M.R., Changeux J.P., Identification of four classes of brain nicotinic receptors using beta2 mutant mice, *J. Neurosci.* 18 (1998), 4461–4472.
- [48] Clarke P., Shwartz R.D., Paul S.M., Pert C.B., Pert A., Nicotinic binding in rat brain: Autoradiographic comparison of [³H]-ACh, [³H]-nicotine and [¹²⁵I] α -bungarotoxin, *J. Neurosci.* 5 (1985) 1307–1313.
- [49] Perry D.C., Kellar K.J., [³H]epibatidine labels nicotinic receptors in rat brain: an autoradiographic study, *J. Pharmacol. Exp. Ther.* 275 (1995) 1030–1034.
- [50] Orr-Urtreger A., Goldner F.M., Saeki M., Lorenzo I., Goldberg L., De Biasi M., Dani J.A., Patrick J.W., Beaudet A., Mice deficient in the alpha7 neuronal nicotinic acetylcholine receptor lack alpha-bungarotoxin binding sites and hippocampal fast nicotinic currents, *J. Neurosci.* 17 (1997) 9165–9171.
- [51] Changeux J.P., Bessis A., Bourgeois J.P., Corringer P.J., Devillers-Thiery A., Eisele J.L., Kerszberg M., Léna C., Le Novère N., Picciotto M., Zoli M., Nicotinic receptors and brain plasticity, *Cold Spring Harb. Symp. Quant. Biol.* 61 (1996) 343–362.
- [52] Treinin M., Chalfie M., A mutated acetylcholine receptor subunit causes neuronal degeneration in *C. elegans*, *Neuron* 14 (1995) 871–877.
- [53] Ohno K., Wang H.L., Milone M., Bren N., Brengman J.M., Nakano S., Quiram P., Pruitt J.N., Sine S.M., Engel A.G., Congenital myasthenic syndrome caused by decreased agonist binding affinity due to a mutation in the acetylcholine receptor epsilon subunit, *Neuron* 17 (1996) 157–170.
- [54] Ohno K., Quiram P.A., Milone M., Wang H.L., Harper M.C., Pruitt J.N., Brengman J.M., Pao L., Fischbeck K.H., Crawford T.O., Sine S.M., Engel A.G., Congenital myasthenic syndromes due to heteroallelic nonsense/missense mutations in the acetylcholine receptor epsilon subunit gene - identification and functional characterization of six new mutations, *Hum. Mol. Genet.* 6 (1997) 753–766.
- [55] Milone M., Wang H.L., Ohno K., Prince R., Fukudome T., Shen X.M., Brengman J.M., Griggs R.C., Sine S.M., Engel A.G., Mode switching kinetics produced by a naturally occurring mutation in the cytoplasmic loop of the human acetylcholine receptor epsilon subunit, *Neuron* 20 (1998) 575–588.
- [56] Engel A.G., Ohno K., Bouzat C., Sine S.M., Griggs R.C., End-plate acetylcholine receptor deficiency due to nonsense mutations in the epsilon subunit, *Ann. Neurol.* 40 (1996) 810–817.
- [57] Witzemann V., Schwarz H., Koenen M., Berberich C., Villarroel A., Wernig A., Brenner H.R., Sakmann B., Acetylcholine receptor epsilon-subunit deletion causes muscle weakness and atrophy in juvenile and adult mice, *Proc. Natl. Acad. Sci. USA* 93 (1996) 13286–13291.
- [58] Ohno K., Hutchinson D.O., Milone M., Brengman J.M., Bouzat C., Sine S.M., Engel A.G., Congenital myasthenic syndrome caused by prolonged acetylcholine receptor channel openings due to a mutation in the M2 domain of the epsilon subunit, *Proc. Natl. Acad. Sci. USA* 92 (1995) 758–762.
- [59] Sine S.M., Ohno K., Bouzat C., Auerbach A., Milone M., Pruitt J.N., Engel A.G., Mutation of the acetylcholine receptor alpha subunit causes a slow-channel myasthenic syndrome by enhancing agonist binding affinity, *Neuron* 15 (1995) 229–239.
- [60] Engel A.G., Ohno K., Milone M., Wang H.L., Nakano S., Bouzat C., Pruitt J.N. 2nd., Hutchinson D.O., Brengman J.M., Bren N., Sieb J.P., Sine S.M., New mutations in acetylcholine receptor subunit genes reveal heterogeneity in the slow-channel congenital myasthenic syndrome, *Hum. Mol. Genet.* 5 (1996) 1217–1227.
- [61] Gomez C.M., Maselli R., Gammack J., Lasalde J., Tamamizu S., Cornblath D.R., Lehar M., McNamee M., Kuncel R.W., A beta-subunit mutation in the acetylcholine receptor channel gate causes severe slow-channel syndrome, *Ann. Neurol.* 39 (1996) 712–723.
- [62] Croxen R., Newland C., Beeson D., Oosterhuis H., Chauplannaz G., Vincent A., Newsom-Davis J., Mutations in different functional domains of the human muscle acetylcholine receptor alpha subunit in patients with the slow-channel congenital myasthenic syndrome, *Hum. Mol. Genet.* 6 (1997) 767–774.
- [63] Milone M., Wang H., Ohno K., Fukudome T., Pruitt J., Bren N., Sine S., Engel A., Slow-channel myasthenic syndrome caused by enhanced activation, desensitization and agonist binding affinity attributable to mutation in the M2 domain of acetylcholine receptor alpha subunit, *J. Neurosci.* 17 (1997) 5651–5665.
- [64] Edelstein S.J., Changeux J.P., Allosteric proteins after thirty years: the binding and state functions of the neuronal alpha7 nicotinic acetylcholine receptors, *Experientia* 52 (1996) 1083–1090.
- [65] Gomez C.M., Maselli R., Gundeck J.E., Chao M., Day J.W., Tamamizu S., Lasalde J.A., McNamee M., Wollmann R.L., Slow-channel transgenic mice – a model of postsynaptic organellar degeneration at the neuromuscular junction, *J. Neurosci.* 17 (1997) 4170–4179.
- [66] Steinlein O.K., Mulley J.C., Propping P., Wallace R.H., Phillips H.A., Sutherland G.R., Scheffer J.E., Berkovic S.F., A missense mutation in the neuronal nicotinic acetylcholine receptor alpha4 subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy, *Nat. Genet.* 11 (1995) 201–203.
- [67] Weiland S., Witzemann V., Villarroel A., Propping P., Steinlein O., An amino acid exchange in the second transmembrane segment of a neuronal nicotinic receptor causes partial epilepsy by altering its desensitization kinetics, *FEBS Lett.* 398 (1996) 91–96.
- [68] Kuryatov A., Gerzanich V., Nelson M., Olale F., Lindstrom J., Mutation causing autosomal dominant nocturnal frontal lobe epilepsy alters Ca²⁺ permeability, conductance, and gating of human alpha4beta2 nicotinic acetylcholine receptors, *J. Neurosci.* 17 (1997) 9035–9047.
- [69] Steinlein O.K., Magnusson A., Stoodt J., Bertrand S., Weiland S., Berkovic S.F., Nakken K.O., Propping P., Bertrand D., An insertion mutation of the CHRNA4 gene in a family with autosomal dominant nocturnal frontal lobe epilepsy, *Hum. Mol. Genet.* 6 (1997) 943–947.
- [70] Lynch J.W., Rajendra S., Pierce K.D., Handford C.A., Barry P.H., Schofield P.R., Identification of intracellular and extracellular domains mediating signal transduction in the inhibitory glycine receptor chloride channel, *EMBO J.* 16 (1997) 110–120.
- [71] Léna C., Changeux J.P., Role of Ca²⁺ ions in nicotinic facilitation of GABA release in mouse thalamus, *J. Neurosci.* 17 (1997) 576–585.

- [72] Orr Urtreger A., Noebels J.L., Goldner F.M., Patrick J., Beaudet A.L., A novel hypersynchronous neocortical EEG phenotype in mice deficient in the neuronal nicotinic acetylcholine receptor (nAChRs) $\alpha 7$ subunit gene, *Am. J. Hum. Genet.* 59 (1996) A53.
- [73] Bierer L.M., Haroutunian V., Gabriel S., Knott P.J., Carlin L.S., Purohit D.P., Perl D.P., Schmeidler J., Kanof P., Davis K.L., Neurochemical correlates of dementia severity in Alzheimer's disease: relative importance of the cholinergic deficits, *J. Neurochem.* 64 (1995) 749-760.
- [74] Perry E.K., Morris C.M., Court J.A., Cheng A., Fairbairn A.F., McKeith I.G., Irving D., Brown A., Perry R.H., Alteration in nicotine binding sites in Parkinson's disease, Lewy body dementia and Alzheimer's disease: possible index of early neuropathology, *Neuroscience* 64 (1995) 385-395.
- [75] Newhouse P.A., Sunderland T., Tariot P.N., Blumhardt C.L., Weingartner H., Mellow A., Murphy D.L., Intravenous nicotine in Alzheimer's disease: a pilot study, *Psychopharmacology* 95 (1988) 171-175.
- [76] Jones G.M., Sahakian B.J., Levy R., Warburton D.M., Gray J.A., Effects of acute subcutaneous nicotine on attention, information processing and short-term memory in Alzheimer's disease, *Psychopharmacology* 108 (1992) 485-494.
- [77] Marchi M., Raiteri M., Nicotinic autoreceptors mediating enhancement of acetylcholine release become operative in conditions of 'impaired' cholinergic presynaptic function, *J. Neurochem.* 67 (1996) 1974-1981.
- [78] Arneric S.P., Sullivan J.P., Decker M.W., Brioni J.D., Bannon A.W., Briggs C.A., Donnelly R.D., Radek R.J., Marsh K.C., Kyncl J., et al., Potential treatment of Alzheimer disease using cholinergic channel activators (ChCAs) with cognitive enhancement, anxiolytic-like, and cytoprotective properties, *Alzheimer Dis. Assoc. Disord.* 2 (1995) 50-61.
- [79] Cosford N.D., Bleicher L., Herbaut A., McCallum J.S., Vernier J.M., Dawson H., Whitten J.P., Adams P., Chavez N.L., Correa L.D., Crona J.H., Mahaffy L.S., Menzaghi F., Rao T.S., Reid R., Sacca A.I., Santori E., Stauderman K.A., Whelan K., Lloyd G.K., McDonald I.A., (S)-(-)-5-ethynyl-3-(1-methyl-2-pyrrolidinyl)pyridine maleate (SIB-1508Y): a novel anti-parkinsonian agent with selectivity for neuronal nicotinic acetylcholine receptors, *J. Med. Chem.* 39 (1996) 3235-3237.
- [80] Lippiello P.M., Bencherif M., Gray J.A., Peters S., Grigoryan G., Hodges H., Collins A.C., RJR-2403: a nicotinic agonist with CNS selectivity II. In vivo characterization, *J. Pharmacol. Exp. Ther.* 279 (1996) 1422-1429.
- [81] Dehaene S., Changeux J.P., The Wisconsin Card Sorting Test: theoretical analysis and modeling in a neuronal network, *Cereb. Cortex* 1 (1991) 62-79.
- [82] Pennartz C., The ascending neuromodulatory systems in learning by reinforcement -comparing computational conjectures with experimental findings, *Brain Res. Brain Res. Rev.* 21 (1996) 219-245.
- [83] Altman J., Everitt B.J., Glautier S., Markou A., Nutt D., Oretti R., Phillips G.D., Robbins T.W., The biological, social and clinical bases of drug addiction: commentary and debate, *Psychopharmacology* 125 (1996) 285-345.
- [84] Rose J.E., Corrigan W.A., Nicotine self-administration in animals and humans - similarities and differences, *Psychopharmacology* 130 (1997) 28-40.
- [85] Pontieri F.E., Tanda G., Orzi F., Di Chiara G., Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs, *Nature* 382 (1996) 255-257.
- [86] Merlo Pich E., Pagliusi S.R., Tessari M., Talbot-Ayer D., Hoof van Huijsduijnen R., Chiamulera C., Common neural substrates for the addictive properties of nicotine and cocaine, *Science* 275 (1997) 83-86.
- [87] Fowler J.S., Volkow N.D., Wang G.J., Pappas N., Logan J., MacGregor R., Alexoff D., Shea C., Schlyer D., Wolf A.P., Warner D., Zezulakova I., Cilento R., Inhibition of monoamine oxidase B in the brains of smokers, *Nature* 379 (1996) 733-736.
- [88] Henningfield J.E., Miyasato K., Jasinski D.R., Cigarette smokers self-administer intravenous nicotine, *Pharmacol. Biochem. Behav.* 19 (1983) 887-890.
- [89] Picciotto M.R., Zoli M., Rimondini R., Léna C., Marubio L.M., Merlo Pich E., Fuxe K., Changeux J.P., Acetylcholine receptors containing the $\beta 2$ subunit are involved in the reinforcing properties of nicotine, *Nature* 391 (1998) 173-177.
- [90] Ochoa E.L., Li L., McNamee M.G., Desensitization of central cholinergic mechanisms and neuroadaptation to nicotine, *Mol. Neurobiol.* 4 (1990) 251-287.
- [91] Pidoplichko V., DeBiasi M., Williams J.T., Dani J.A., Nicotine activates and desensitizes midbrain dopamine neurons, *Nature* 390 (1997) 401-404.
- [92] Egan T.M., North R.A., Actions of acetylcholine and nicotine on rat locus coeruleus neurons in vitro, *Neuroscience* 19 (1986) 565-567.
- [93] Zhu J.J., Ulrich D.J., Nicotinic receptor-mediated responses in relay cells and interneurons in the rat lateral geniculate nucleus, *Neuroscience* 80 (1997) 191-202.
- [94] Fenster C.P., Rains M.F., Noerager B., Quick M.W., Lester R.A., Influence of subunit composition on desensitization of neuronal acetylcholine receptors at low concentrations of nicotine, *J. Neurosci.* 17 (1997) 5747-5759.
- [95] Futami T., Takakusaki K., Kitai S.K., Glutamatergic and cholinergic inputs from the pedunculopontine tegmental nucleus to dopamine neurons in the substantia nigra pars compacta, *Neuroscience Res.* 21 (1995) 331-342.
- [96] Yeomans J., Baptista M., Both nicotinic and muscarinic receptors in ventral tegmental area contribute to brain-stimulation, *Pharmacol. Biochem. Behav.* 57 (1997) 915-921.
- [97] Ivanova S., Greenshaw A.J., Nicotine-induced decreases in VTA electrical self-stimulation thresholds: blockade by haloperidol and mecamylamine but not scopolamine or ondansetron, *Psychopharmacology* 134 (1997) 187-192.
- [98] Epping-Jordan M.P., Watkins S.S., Koob G.F., Markou A., Dramatic decreases in brain reward function during nicotine withdrawal, *Nature* 393 (1998) 76-79.
- [99] Nisell M., Nomikos G.G., Svensson T.H., Nicotine dependence, midbrain dopamine systems and psychiatric disorders, *Pharmacol. Toxicol.* 76 (1995) 157-162.
- [100] Levin E.D., Wilson W., Rose J.E., McEvoy J., Nicotine-haloperidol interactions and cognitive performance in schizophrenics, *Neuropsychopharmacology* 15 (1996) 429-436.
- [101] Shytle R.D., Silver A.A., Philipp M.K., McConville B.J., Sanberg P.R., Transdermal nicotine for Tourette's syndrome, *Drug Dev. Res.* 38 (1996) 290-298.
- [102] Levin E.D., Connors C.K., Sparrow E., Hinton S.C., Erhardt D., Meck W.H., Rose J.E., March J., Nicotine effects on adults with attention-deficit/hyperactivity disorder, *Psychopharmacology* 123 (1996) 55-63.
- [103] Fagerstrom K.O., Pomerleau O., Giordani B., Stelson F., Nicotine may relieve symptoms of Parkinson's disease, *Psychopharmacology* 116 (1994) 117-119.

- [104] Clemens P., Baron J.A., Coffey D., Reeves A., The short term effect of nicotine chewing gum in patients with Parkinson's disease, *Psychopharmacology* 117 (1995) 253–256.
- [105] Leonard S., Adams C., Breese C.R., Adler L.E., Bickford P., Byerley W., Coon H., Griffith J.M., Miller C., Myles W.M., Nagamoto H.T., Rollins Y., Stevens K.E., Waldo M., Freedman R., Nicotinic receptor function in schizophrenia, *Schizophr. Bull.* 22 (1996) 431–445.
- [106] Freedman R., Coon H., Mylesworsley M., Orr Urtreger A., Olincy A., Davis A., Polymeropoulos M., Holik J., Hopkins J., Hoff M., Rosenthal J., Waldo M.C., Reimherr F., Wender P., Yaw J., Young D.A., Breese C.R., Adams C., Patterson D., Adler L.E., Kruglyak L., Leonard S., Byerley W., Linkage of a neurophysiological deficit in schizophrenia to a chromosome 15 locus, *Proc. Natl. Acad. Sci. USA* 94 (1997) 587–592.
- [107] McGehee D.S., Heath M.J., Gelber S., Devay P., Role L.W., Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors, *Science* 269 (1995) 1692–1696.
- [108] Gray R., Rajan A.S., Radcliffe K.A., Yakehiro M., Dani J.A., Hippocampal synaptic transmission enhanced by low concentrations of nicotine, *Nature* 383 (1996) 713–716.
- [109] Chen D.N., Patrick J.W., The alpha-bungarotoxin-binding nicotinic acetylcholine receptors from rat brain contains only the alpha7 subunit, *J. Biol. Chem.* 272 (1997) 24024–24029.
- [110] Lefkowitz R.J., Cotecchia S., Samama P., Costa T., Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins, *Trends Pharmacol. Sci.* 14 (1993) 303–307.
- [111] Gomez C.M., Gammack J.T., A leucine-to-phenylalanine substitution in the acetylcholine receptor ion channel in a family with the slow-channel syndrome, *Neurology* 45 (1995) 982–985.
- [112] Winzer-Serhan U.H., Leslie F.M., Codistribution of nicotinic acetylcholine receptor subunit alpha3 and beta4 mRNAs during rat brain development, *J. Comp. Neurol.* 386 (1997) 540–554.

Dissection of active zones at the neuromuscular junction by EM tomography

Mark Harlow^a, David Ress^a, Abraham Koster^{b**}, Robert M. Marshall^a, Mikael Schwarz^a,
Uel Jackson McMahan^{a*}

^a*Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305, USA*

^b*Department of Structural Biology, Max-Planck Institute for Biochemistry, Martinsried, Germany*

Abstract — We used EM tomography to examine the fine structure of the apparently amorphous electron dense material that is seen at active zones of axon terminals when viewed by conventional 2D electron microscopy. Serial 1-nm optical slices from 3D reconstructions of individual thin tissue sections reveal that the material is composed of an interconnecting network of elongate components directly linked to synaptic vesicles and the presynaptic membrane. Each vesicle at the active zone that lies adjacent to the presynaptic plasma membrane has several such connections. Information provided by reconstruction data may be useful in generating experiments aimed at understanding the mechanisms involved in the docking of synaptic vesicles and their exocytosis during synaptic transmission. (©Elsevier, Paris)

Résumé — **Dissection des zones actives à la jonction neuromusculaire par tomographie ME.** Nous avons utilisé la tomographie ME pour examiner aux électrons la structure fine du matériel dense apparemment amorphe qui est observé au niveau des zones actives des terminaisons axonales en microscopie électronique conventionnelle 2D. Des reconstructions 3D obtenues à partir de coupes sériées de 1 nm font apparaître un matériel constitué d'un réseau connectif de composés directement liés aux vésicules synaptiques et à la membrane présynaptique. (©Elsevier, Paris)

neuromuscular junctions / active zones (3D fine structure of) / EM tomography

1. Introduction

Conventional 2D electron microscope images of neuromuscular junctions made from thin sections of fixed, metal stained and plastic embedded tissue reveal that the synaptic vesicles in the axon terminal are focused on patches of electron dense material lining the cytoplasmic surface of the presynaptic membrane [1]. It is along side these patches that the vesicles undergo exocytosis to release their acetylcholine into the synaptic cleft during synaptic transmission [1, 3]. Thus, the patches of dense material and their associated vesicles mark the 'active zones' [1] of the presynaptic membrane. The close and constant positioning of synaptic vesicles adjacent to the dense material has led to the concept that vesicles are directly associated with the material prior to exocytosis and that such an association is necessary for normal synaptic transmission to occur. Indeed, one would expect the recently identified proteins [11] thought to be involved in vesicle docking and exocytosis to be at or near the dense material. The dense material in 2D images of thin

sections does often appear to have substructure, but because the dimensions of the components are considerably less than the thickness of a section, their outlines are usually indistinct and they are not obviously organized. Moreover, although the dense material seems to contact the vesicles and plasma membrane, the likelihood of such connections is made uncertain by the absence of z axis information; e.g., vesicles and portions of the material that seem to contact each other in the x-y axis of even the thinnest sections that can be cut (30–50 nm) might actually lie in different z axis planes.

We have begun to examine the patches of active zone material in 3D reconstructions of sections from muscles prepared by the same techniques used for conventional 2D electron microscopy. The reconstructions are generated by electron microscope tomography. This is part of a long range EM tomography study of the entire synaptic apparatus, including the basal lamina of the synaptic cleft and the band of electron dense material that lines the cytoplasmic surface of the postsynaptic membrane. Like the active zone material, these structures appear nearly amorphous in 2D images and they are known to contain aggregates of proteins that are involved in synaptic function. If this material has relationships and organization that are obscured in 2D images by the absence of z axis information, they might be ap-

* Correspondence and reprints.

** Present address: Department of Molecular Cell Biology, Utrecht University, Utrecht, the Netherlands.

parent in 3D reconstructions. The exposure of such architecture might then make it possible to accurately map at the nanometer level the relative position of synapse specific proteins within it through the use of, for example, antibodies, toxins, or gene deletions. Such information is crucial for a thorough understanding of the mechanisms involved in the assembly of synaptic apparatus and how each of its components functions.

EM tomography involves the integration of multiple 2D images of a section made at different degrees of tilt relative to the electron beam [2]. It has been extensively used by others over the last decade to determine the structure of isolated macromolecules, organelles and viruses [7, 12–14]. It has also been used on tissue sections for examining the contractile apparatus of muscle fibers [9] and the membrane arrangement in mitochondria [8]. Here we present our initial findings on the structure of the active zone material and its relationship to synaptic vesicles at the frog's neuromuscular junction, which has long been a model for studies on synaptic structure and function [1, 3–5].

2. Materials and methods

2.1. Fixation, staining, embedding and microscopy for the reconstruction

Immediately after killing a *Rana pipiens*, the skin overlying the thin cutaneous pectoris muscles was slit open and tissue paper soaked in 1% phosphate buffered glutaraldehyde (240 mOsM) was placed directly on the muscles. 30 min later the muscles were removed and bathed in the same solution for 30 min more. After rinsing them with phosphate buffer, the muscles were immersed in phosphate buffered osmium tetroxide, rinsed in distilled water, treated with saturated aqueous uranyl acetate, dehydrated in ethanol and propylene oxide and embedded in Epon. Thin sections having silver interference colors were mounted on single slot Formvar coated grids and stained with uranyl acetate followed by lead citrate. They were then coated with dilute 10 nm colloidal gold particles to provide fiducials for the alignment of tilt images. The data were collected with a Philips CM200 electron microscope, which was equipped with a FEG, cryoholder and 1024 × 1024 CCD. The cryoholder was filled with liquid nitrogen which maintained the specimen at $\sim -186^\circ\text{C}$ to inhibit beam-induced specimen shrinkage during data collection. Tilt ima-

ges were collected at 2° intervals to plus and minus 66° from horizontal. The images were made at a microscope magnification of $\times 34\,000$; a single pixel on the CCD corresponded to 0.26 nm. The total beam dosage during data collection was $\sim 350\,000\text{ e}^-/\text{nm}^2$.

2.2. Filtering, alignment and reconstruction

Each 2D image in a tilt series was first filtered to reduce noise using a steerable pyramid-scheme [10]. Next, the images were coaligned using an automatic computer algorithm that detects the location of the fiducial gold on each image. The accuracy of alignment is a major determining factor in the spatial resolution in the volume; the alignment accuracy on the data set presented here is 1 nm RMS. Finally, weighted back projection was used to convert the coaligned set of 2D images into a 3D volume.

3. Results and discussion

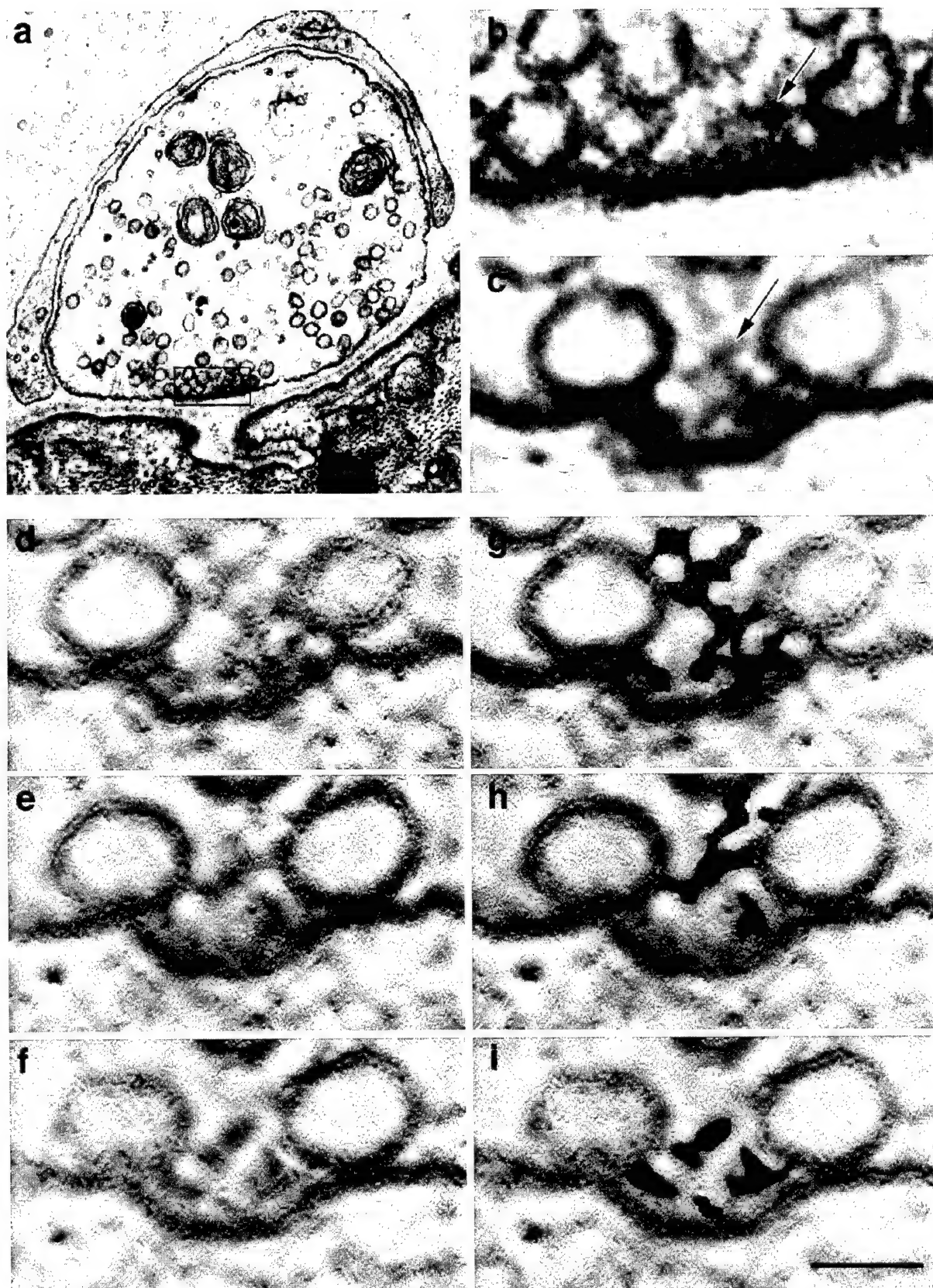
Figure 1a is a 2D image of a section from a neuromuscular junction made by conventional electron microscopy. It shows the synaptic vesicle-containing axon terminal and the surface of the muscle fiber characterized by its infolding of the postsynaptic plasma membrane. The plasma membranes of the terminal and muscle fiber are separated by the basal lamina-filled synaptic cleft. This axon terminal profile has a single active zone (box). Typically, the active zone is situated just opposite the infolding in the muscle fiber's plasma membrane.

Figure 1b is a higher magnification view of the active zone in figure 1a; the field corresponds to that outlined by the box in figure 1a. The patch of electron dense active zone material (arrow) is amorphous and appears to bear a close relationship to the vesicles associated with it, some of which are also closely associated with the plasma membrane.

Figure 1c is a 2D image of a 3D reconstruction of a section of a neuromuscular junction made by EM tomography. It was formed by summing the central 40 nm of the volume along the z axis. The field is similar to that in figure 1b; a synaptic vesicle closely apposed to the presynaptic membrane is situated on each side of the active zone material (arrow). By counting voxels in the z axis of the full reconstruction, it was determined that the total thickness of

Figure 1. Electron dense material at active zones of the frog's neuromuscular junction in images made by conventional 2D EM and by EM tomography. **a.** Low power view of an axon terminal and muscle fiber surface made by 2D EM. Box outlines active zone. **b.** Higher power view of active zone in box in **a** showing electron dense active zone material (arrow). **c.** 40 nm thick reconstruction of an active zone made by EM tomography showing electron dense active zone material (arrow). **d, e, f.** 1 nm thick optical sections from different z axis planes of the reconstruction shown in **c**. **g, h, i.** Same optical sections as in **d, e, f** with certain components of the active zone material are emphasized. Scale bar, **a**, 400 nm; **b**, 88 nm; **c–i**, 50 nm.





the section was 50 nm; the top and bottom 5 nm were discarded because they contained obvious artifact. The contrast and delineation of the plasma and vesicle membranes in the reconstruction compares favorably to that of the same structures in conventional 2D images (*figure 1b*) as does the active zone material.

Figure 1d, e and *f* show three optical slices, 1 nm (4 voxels) thick, from the reconstruction in *figure 1c*. All are in the x-y plane but each is from a different level in the z-axis. They illustrate what we have now observed at all of several active zones so far examined in this way: 1) the active zone material is composed of elongate interconnecting structures; 2) the structures abut synaptic vesicles and the presynaptic plasma membrane; and 3) each vesicle is contacted by several such structures. *Figure 1g, h* and *i* are duplicates of *figure 1d, e* and *f*; the gray-scale density of some components of the active zone material was selectively increased to facilitate viewing.

Although images from the reconstruction leave little doubt that the patch of active zone material consists of a complex arrangement of measureable subcomponents and that certain of these components are linked to synaptic vesicles and presynaptic plasma membrane, we have not yet identified a pattern of component shapes and organization common to all patches of active zone material. This might be expected if, for example, the active zone material associated with each vesicle were a functional unit. In order to be able to recognize any such patterns within the complexity of structures at an active zone, we are currently developing volume segmentation methods to extract and quantify shapes and relationships. It may well be that aldehyde fixation, as used for the reconstruction demonstrated here, distorts the material at different active zones in a random way, hindering recognition of a common organizational scheme. Accordingly we have also begun to reconstruct active zones in muscles prepared by 'ultra-rapid' freezing and acetone freeze-substitution, which provide better preservation of certain cellular structures than aldehyde fixation [6].

Acknowledgments

The authors are grateful to Professor Wolfgang Baumeister, Director of the Department of Structural Biology at the Max-Planck Institute for Biochemistry in Martinsried, Germany, and to members of his department for the use of the

department's Philips CM200 electron microscope and providing helpful discussions. Supported by NIH training grant 5T32 NS 07158 (M.H. and D.R.), D9FTy2/4-1,2 (A.K.), the M.A. Antelman Fund and NIH research grant 2R01 NS14506 (U.J.M.).

References

- [1] Couteux R., Pecot-Dechavassine M., Vesicules synaptiques et poches au niveau des 'zones actives' de la jonction neuromusculaire, *C.R. Acad. Sci. Ser. D* 271 (1970) 2346-2349.
- [2] Franck J., Electron tomography: three-dimensional structures from electron micrographs, Plenum Press, New York, 1992.
- [3] Heuser J.E., Reese T.S., Dennis M.J., Jan L., Jan Y., Evans L., Synaptic vesicle exocytosis captured by quick-freezing and correlated with quantal transmitter release, *J. Cell Biol.* 81 (1979) 275-300.
- [4] Katz B., The release of neural transmitter substances, C.C. Thomas, Springfield, Illinois, 1969.
- [5] McMahan U.J., Wallace B., Molecules in basal lamina that direct the formation of synaptic specializations at neuromuscular junctions, *Dev. Neurosci.* 11 (1989) 227-247.
- [6] Moor H., Theory and practice of high pressure freezing in cryotechniques, in: Steinbrecht R.A., Zierod K. (Eds.), *Biological Electron Microscopy*, Springer-Verlag, Berlin, 1987, pp. 175-191.
- [7] Moritz M., Braunfeld M., Fung J., Sedat J., Alberts B., Agard D., Three-dimensional structural characterization of centrosomes from early drosophila embryos, *J. Cell Biol.* 130 (1995) 1149-1159.
- [8] Perkins G., Renken C., Martone M., Young S., Ellisman M., Electron tomography of neuronal mitochondria: three-dimensional structure and organization of cristae and membrane contacts, *J. Struct. Biol.* 119 (1997) 260-272.
- [9] Schroeter J., Bretauiere J., Sass R., Goldstein M., Three-dimensional structure of the Z band in a normal mammalian skeletal muscle, *J. Cell Biol.* 133 (1996) 571-583.
- [10] Simoncelli E., Freeman W., Adelson E., Heeger D., Shiftable multiscale transforms, *IEEE Trans. Info. Theory* 38 (1992) 587-607.
- [11] Sudhof T., 'The synaptic vesicle cycle' a cascade of protein-protein interactions, *Nature* 375 (1995) 645-653.
- [12] Whittaker M., Milligan R., Conformational changes due to calcium-induced calmodulin dissociation in brush border myosin I-decorated F-actin revealed by cryoelectron microscopy and image analysis, *J. Mol. Biol.* 269 (1997) 548-557.
- [13] Zhou Z., Prasad B., Jakana J., Rixon F., Chiu W., Protein subunit structures in the herpes simplex virus capsid from 400 kV spot-scan electron cryomicroscopy, *J. Mol. Biol.* 242 (1994) 456-469.
- [14] Zwickl P., Grziwa A., Puhler G., Dahlmann B., Lottspeich F., Baumeister W., Primary structure of the thermoplasma proteasome and its implications for the structure, function, and evolution of the multicatalytic proteinase, *Biochemistry* 31 (1992) 964-972.

Toxins selective for subunit interfaces as probes of nicotinic acetylcholine receptor structure

Palmer Taylor^a, Hitoshi Osaka^a, Brian E. Molles^a, Naoya Sugiyama^{a*}, Pascale Marchot^{a**},
Elizabeth J. Ackermann^a, Siobhan Malanya^a, Joseph J. McArdle^b, Steven M. Sine^c,
Igor Tsigelny^a

^aDepartment of Pharmacology, 0636 University of California, San Diego, La Jolla, CA 92093, USA

^bDepartment of Pharmacology and Physiology, UMDNJ – New Jersey Medical School, Newark, NJ 07103-2714, USA

^cDepartment of Physiology & Biophysics, Mayo Clinic Foundation, Rochester, MN 55905, USA

Abstract — The pentameric structure of the nicotinic acetylcholine receptor with two of the five subunit interfaces serving as ligand binding sites offers an opportunity to distinguish features on the surfaces of the subunits and their ligand specificity characteristics. This problem has been approached through the study of assembly of subunits and binding characteristics of selective peptide toxins. The receptor, with its circular order of homologous subunits ($\alpha\gamma\alpha\delta\beta$), assembles in only one arrangement, and through mutagenesis, the residues governing assembly can be ascertained. Selectivity of certain toxins is sufficient to readily distinguish between sites at the $\alpha\gamma$ and $\alpha\delta$ interfaces. By interchanging residues on the γ and δ subunits, and ascertaining how they interact with the α -subunit, determinants forming the binding sites can be delineated. The α -conotoxins, which contain two disulfide loops and 12–14 amino acids, show a 10 000-fold preference for the $\alpha\delta$ over the $\alpha\gamma$ subunit interface with $\alpha\epsilon$ falling between the two. The waglerins, as 22–24 amino acid peptides with a single core disulfide loop, show a 2000-fold preference for $\alpha\epsilon$ over the $\alpha\gamma$ and $\alpha\delta$ interfaces. Finally, the 6700 Da short α -neurotoxin from *N. mossambica mossambica* shows a 10 000-fold preference for the $\alpha\gamma$ and $\alpha\delta$ interfaces over $\alpha\epsilon$. Selective mutagenesis enables one to also distinguish α -neurotoxin binding at the $\alpha\gamma$ and $\alpha\delta$ subunits. This information, when coupled with homology modeling of domains and site-directed residue modification, reveals important elements of receptor structure and conformation. (©Elsevier, Paris)

Résumé — Liaison sélective de toxines aux interfaces entre sous-unités : structure du récepteur cholinergique. La structure pentamérique du récepteur nicotinique, dans laquelle deux des cinq interfaces entre sous-unités servent de sites de liaison pour l'acétylcholine, offre la possibilité de distinguer les caractéristiques des deux sites et leur spécificité pour les ligands. Nous avons abordé l'étude de l'assemblage des sous-unités par la liaison de toxines peptidiques sélectives. Le récepteur s'assemble d'une seule manière avec un ordre circulaire unique de sous-unités ($\alpha\gamma\alpha\delta\beta$), et il est possible d'identifier les résidus qui contrôlent ces interactions par mutagenèse dirigée. La sélectivité de certaines toxines est suffisante pour distinguer clairement les sites des interfaces $\alpha\gamma$ et $\alpha\delta$. En échangeant des résidus entre les sous-unités α et δ , et en analysant leurs associations avec la sous-unité α , on peut définir les déterminants des sites de liaison. Les conotoxines α formées de 12–14 acides aminés et contenant deux ponts disulfure se lient 10 000 fois mieux au site $\alpha\gamma$, et de façon intermédiaire à $\alpha\epsilon$. Les wagherines, formées de 20–24 acides aminés et possédant un seul pont disulfure se lient 2 000 fois mieux à $\alpha\epsilon$ qu'à $\alpha\gamma$ et $\alpha\delta$. Finalement, la neurotoxine α courte de 6 700 Da de *Naja mossambica mossambica* présente une préférence de 10 000 fois pour les interfaces $\alpha\gamma$ et $\alpha\delta$, par rapport à $\alpha\epsilon$. La mutagenèse permet aussi de distinguer la liaison de l' α -neurotoxine aux sites $\alpha\gamma$ et $\alpha\delta$. Ces informations, associées à la modélisation des domaines par homologie et la modification de résidus par mutagenèse dirigée, donnent d'importants éléments pour comprendre la structure et la conformation du récepteur. (©Elsevier, Paris)

homologous subunits / nicotinic acetylcholine receptor / ligand binding sites

The nicotinic acetylcholine receptor (nAChR) is a circular pentamer composed of four homologous subunits with the stoichiometry of $\alpha_2\beta\gamma\delta$ arranged around a central channel [4, 8]. In the mature neuromuscular junction of mammals, the ϵ -subunit replaces γ with minimal changes in specificity for acetylcholine and the alkaloid antagonists. The two

binding sites exist at the $\alpha\gamma$ (or $\alpha\epsilon$) and $\alpha\delta$ interfaces; simultaneous occupation of the two sites gives rise to channel opening. Occupation of one of the two sites by an antagonist is sufficient to result in very low probabilities of channel opening, even in the presence of agonist.

The close sequence identity of the γ , δ and ϵ subunits and the fact that homologous residues should exist in the same three-dimensional space in a oligomer composed of related subunits offer the possibility of residue substitution to examine the relation between structure and specificity of binding events occurring at the subunit interface.

* Present address: Department of Psychiatry, Yokohama City University, Yokohama, Japan

** Present address: CNRS, Institut Fédératif de Recherche Jean-Roché, Université de la Méditerranée, Marseille cedex 20, France

Our previous studies have examined several determinants of subunit assembly, both in terms of a preferential subunit order [9] and kinetics of assembly [9, 16]. These studies have identified and confirmed the likely presence of particular residues at the subunit interface. The requirements of a specific subunit order revealed that Lys 145 and Lys 150, found in the δ subunit but not in γ and ϵ , form the structural basis for the δ subunit's resistance to be located between the two α -subunits [9]. Moreover, residues 145 and 150 reside on the non-ligand binding surface of the δ subunit.

When studying whether subunit glycosylation would affect α -conotoxin association as it does for the larger α -neurotoxins (figure 1), we noted that α -conotoxin M1 only associated with high affinity at one of the two subunit interfaces [10]. More detailed studies showed that it bound to the $\alpha\delta$ subunit with 30 000 times the affinity of $\alpha\gamma$. $K_{D,\alpha\delta} = 6.2 \times 10^{-10}$ M and $K_{D,\alpha\gamma} = 2.0 \times 10^{-5}$ M (figure 2A). Preferential

binding to the $\alpha\delta$ site was also demonstrated by transfection of $\alpha\delta$ or $\alpha\gamma$ subunit pairs into null cells and examining ^{125}I - α -conotoxin M1 (Tyr 12) binding to the isolated subunit pairs in permeabilized cells [17]. An extensive set of studies initially with chimeras of γ and δ subunit sequences followed by site-specific mutants revealed that three regions in the γ and δ subunits, corresponding to residues 34, 111 and 172 in γ , form the basis for subunit specificity; the residues are likely found at the γ and δ interfaces of the two binding sites [15]. Studies on the α -subunit, whose opposing face should constitute the corresponding portion of the binding site showed that positions in the vicinity of residues 93, 152–154 and 180–200 were contributory to ligand binding [17]. These general regions had been identified previously through photolytic labeling with agonists [5, 6] and site-specific mutagenesis [12, 14, 18]. The influence of combined $\alpha + \delta$ and $\alpha + \gamma$ subunit mutations appeared additive in terms of free energy changes [17].

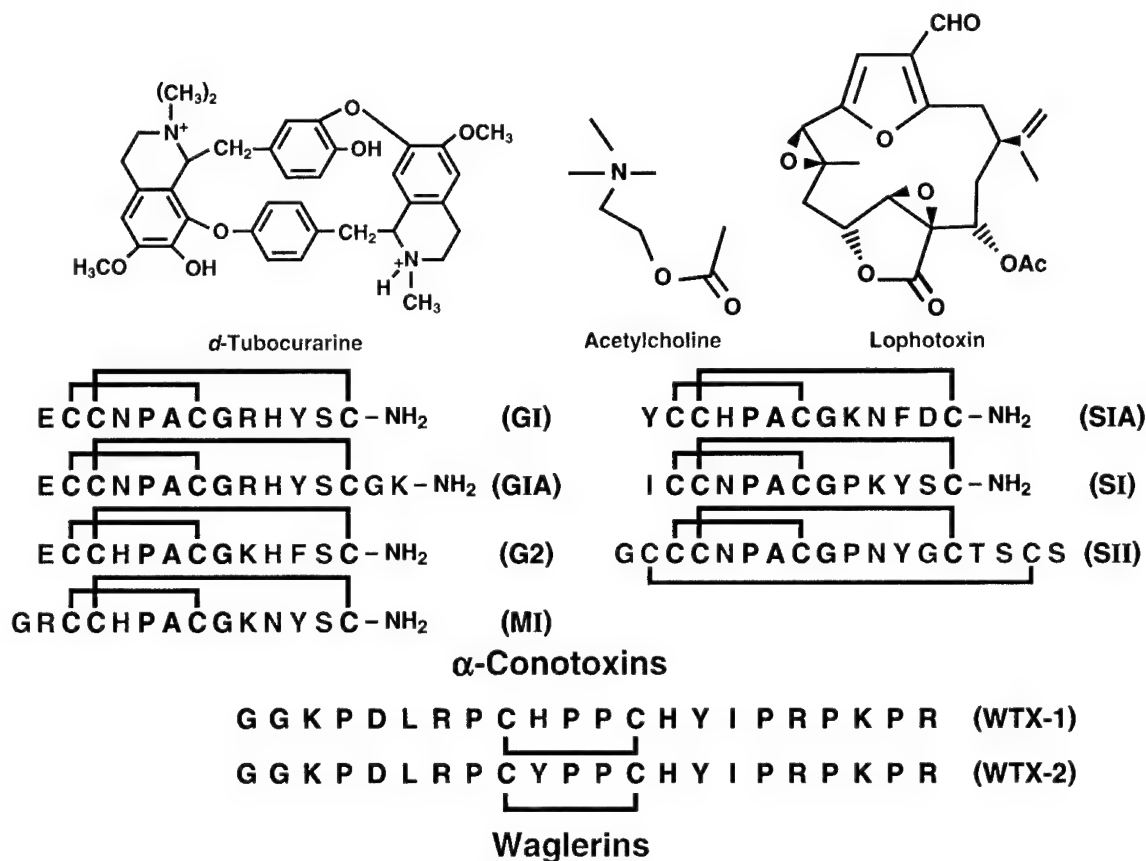


Figure 1. Structures of acetylcholine and various antagonists of the nicotinic receptor. *d*-Tubocurarine is an alkaloid from a plant source, lophotoxin is found in coral of the genus *Lophogorgia*, α -conotoxins are from a Pacific snail, *Conus* sp., and waglerins are from the pit viper, *Trimeresurus wagleri*. Not shown are the 65–75 amino acid, three fingered α -neurotoxin peptides such as α -bungarotoxin and *Naja mossambica mossambica* I α -toxin.

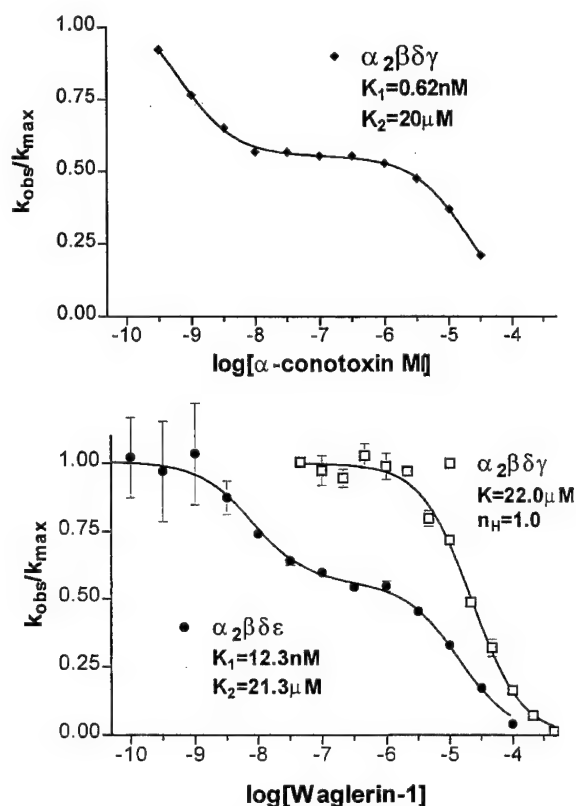


Figure 2. Measurement of small peptide antagonist binding to nicotinic receptors by competition with the initial rate of α -bungarotoxin binding. **A.** Binding of α -conotoxin M1 to the nicotinic receptor expressed as $\alpha_2\beta\gamma\delta$ after prior transfection of the cDNAs encoding the α , β , γ and δ subunits into HEK-293 cells. **B.** Binding of waglerin 1 to the nicotinic receptor expressed as $\alpha_2\beta\epsilon\delta$. Dissociation constants are calculated from the competition curves assuming an equal distribution of binding sites.

While most mutations of the α subunit affected α -conotoxin M1 binding equally at the two sites, certain mutations in the common α -subunit only or primarily affected α -conotoxin binding at one of the two interfaces. For example, the mutations $\alpha Y93F$ and $\alpha V188K$ enhanced and reduced α -conotoxin M1 affinity, respectively, but only at the low affinity, $\alpha\gamma$ site. In contrast, the mutation $\alpha D152N$ only affected α -conotoxin M-1 affinity at the high affinity, $\alpha\delta$ site. This would indicate that the actual orientation of the α -conotoxins, when bound at the $\alpha\gamma$ and $\alpha\delta$ interfaces, are not identical, a point to which we will return later.

A low molecular mass family of toxins, the waglerins, appeared to be a second candidate for these specificity considerations since the waglerins were

shown to be toxic to adult mice [20] (containing ϵ and δ subunits), but not to embryonic and newborn animals (containing γ and δ subunits) (McArdle J.J., personal communication). Since binding at one subunit interface is sufficient for functional antagonism, toxicity should arise from a preferential interaction at the $\alpha\epsilon$ interface. That this is the case is revealed by expression of receptors of compositions $\alpha\epsilon\alpha\delta\beta$ and $\alpha\gamma\alpha\delta\beta$ (figure 2b). Studies with chimeras of ϵ sequence with either γ or δ sequences reveal that two areas corresponding to residues 57–61 and again 172–174 in ϵ are responsible for the specificity of the waglerins (Molles et al., abstract, this conference).

Waglerins also show greater toxicity in mice than rats. Rat and mouse ϵ -subunits only differ by 10 residues in their 210 residue extracellular domain. The analysis, through site specific mutagenesis, shows residues that reside at positions 115 and 59 in the ϵ and γ subunits contribute to the differences between mouse and rat in binding waglerin 1.

A third toxin, *Naja mossambica mossambica* (N.m.m.I toxin), an α -neurotoxin with a molecular mass of 6700, reveals a selectivity for the $\alpha\gamma$, $\alpha\delta$ and $\alpha\epsilon$ binding interfaces that is precisely the opposite of waglerin. The α -neurotoxins of this family were the primary ligands which enabled the identification and initial purification of the nicotinic receptor [4, 11]. Early chemical cross-linking studies involving the α -toxins with the *Torpedo* receptor showed likely contact surfaces with both the α and the γ/δ subunits [13]. The extensive mutagenesis studies of Menez and colleagues [19] have shown many of the α -toxin residue positions involved in the multipoint attachment. Typically the α -toxins, such as α -bungarotoxin and the cobra α -toxins, show near equivalent dissociation constants for their two binding sites in the intact receptor; this is also the case for N.m.m.I toxin with the mouse $\alpha_2\beta\gamma\delta$ receptor where we observe equivalent binding constants whether measured directly or by competition [1]. Surprisingly, the $\alpha_2\beta\epsilon\delta$ combination shows high and low affinity binding sites with N.m.m. α -toxin; hence the ϵ subunit interface is resistant to forming a high affinity complex (Osaka et al., this volume). Studies with chimeras and site-specific mutants pinpoint residues 175 and 176 as influencing N.m.m.I toxin binding, a region just beyond the 172 and 174 residues responsible for specificity of the shorter toxins, α -conotoxin and waglerin 1. Mutations of P175T and E176A substituted into a γ -subunit template yield a low affinity for N.m.m.I α -toxin, equivalent to that of the ϵ subunit. The inverse substitutions T175P and A176E in an ϵ -template give rise to a return of the high affinity. This points to a common region in the γ , δ and ϵ subunits with

which the α -toxin associates, and it should be possible to identify the complementary α -toxin residues presumably in apposition with this region on the receptor.

We have also embarked on studies where we have examined pair-wise interactions between residues on the α subunit of the receptor and the α -toxin [2]. Since Coulombic forces are long range, electrostatic forces lacking specific orientational constraints, we have begun with charged substitutions on both the receptor and α -toxin in order to examine residue proximity. To date, we have examined residues on loop II (K27, R33, R36) and loop III (K47) and residues on the receptor α -subunit between 180 and 200. Figure 3 shows the influence of three toxin mutations on N.m.m.I binding to the receptor. In the case of two of the mutations, shallow curves are observed showing that the binding constants are no longer the same. K_D 's shown in the legend assume equal population of two sites. If the $\alpha\delta$ site is protected with α -conotoxin M1, only 50% of the sites remain and the binding curve steepens. The calculated dissociation constant for the unprotected site corresponds to $K_{D\alpha\gamma}$ and should reflect one of the two dissociation constants calculated from figure 3 [1]. Hence, toxin modification (figure 3) and certain mo-

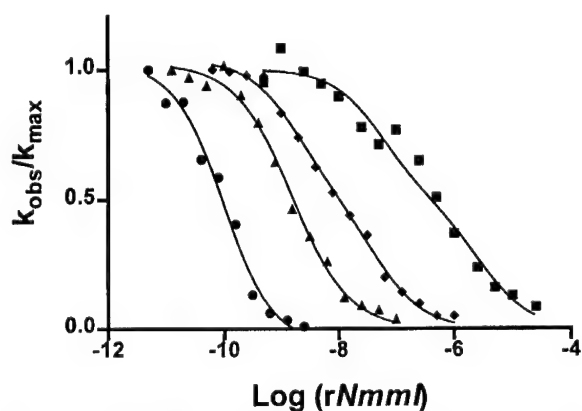


Figure 3. Equilibrium binding of wild-type rNmml and rNmml mutants to wild-type nAChR. Binding of either wild-type rNmml (●), K47A (▲), K27E (◆), or R33E (■) was measured as the fractional reduction in the initial rates of [125]- α -bungarotoxin binding in the absence of rNmml (k_{max}). The curves for wild-type $K_D = 0.14$ nM and for K47A $K_D = 1.5$ nM are least-squares fits to the Hill equation with $n_H = 1.0$. The two sites behave equivalently for the toxins. The curves for K27E and R33E are least-squares fits to two sites present in equal populations. The dissociation constants were determined as: K27E, $K_{D\alpha, \delta} = 1.8$ nM; $K_{D\alpha, \gamma} = 54$ nM; R33E, $K_{D\alpha, \delta} = 95$ nM; $K_{D\alpha, \gamma} = 2.2$ μ M. The assignment of γ or δ to the site comes from α -conotoxin protection experiments as described in the text (modified from Ackermann and Taylor [1]).

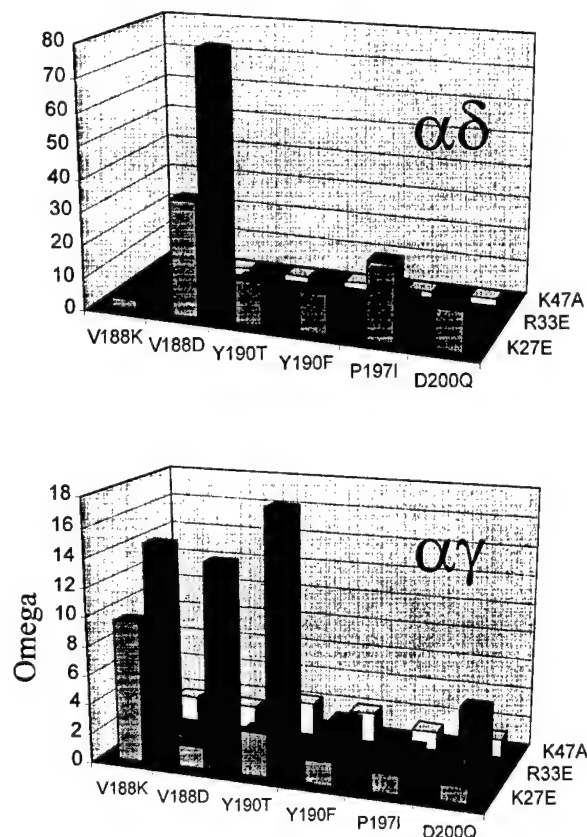


Figure 4. Plot of the Σ values calculated according to equations below for each of the mutant pairs at the $\alpha\delta$ binding site (upper panel) or $\alpha\gamma$ binding site (lower panel). Note that the scale for Σ values differs between the two plots. The value for the R33E/V188D pair at the $\alpha\gamma$ site is 12, with the exact number not determined due to the very large loss in the overall binding affinity.

$$\Omega = \frac{K_D(WT-T, WT-R) \cdot K_D(MT-T, MT-R)}{K_D(MT-T, WT-R) \cdot K_D(WT-T, MT-R)}$$

where the subscripts WT and MT designate wild-type and mutant and T and R designate toxin and nAChR. The pairwise interaction free energy may be calculated from: $\Delta\Delta G_{int} = RT \ln \Omega$ (adapted from Ackermann et al. [2]).

difications of the receptor in the α -subunit (not shown) reveal a non-equivalence in the binding sites. This also indicates that α -toxin binding orientations may not be equivalent at the two sites.

A compilation of interaction energies from the respective K_D values should delineate pair-wise toxin-receptor interactions enabling one to identify interactive (figure 4) and proximal residues on the two molecules in the complex [2]. Minimally, the

position of the α -toxin loops with respect to the subunit faces should emerge from this approach. Second, we have built a model of the extracellular portion of the receptor based on sequence homology of a limited segment of 100 residues with a family of copper binding proteins and residue positions inferred from mutagenesis and site directed labeling [21]. A future challenge is presented in the fitting of the three peptide toxins within the framework of the model structure. Although such an approach will not achieve structural detail comparable to direct physical measurements, the X-ray crystallographic structure of the homologous peptide, fasciculin, with its target acetylcholinesterase provides an interesting template for understanding how the three loops of these toxins interact with a macromolecular target surface [3, 7].

Acknowledgment

Supported by USPHS Grant GM 18360 to P.T.

References

- [1] Ackermann E.J., Taylor P., Non-identity of the α -neurotoxin binding sites on the nicotinic acetylcholine receptor revealed by modification of neurotoxin and receptor structures, *Biochemistry* 36 (1997) 12836–12844.
- [2] Ackermann E.J., Ang E.T.-M., Kanter J., Tsigelny I., Taylor P., Identification of pairwise interactions in the α -neurotoxin-nicotinic acetylcholine receptor complex, *J. Biol. Chem.* 273 (1998) 10958–10954.
- [3] Bourne Y., Taylor P., Marchot P., Acetylcholinesterase inhibition by fasciculin: Crystal structure of the complex, *Cell* 83 (1995) 503–512.
- [4] Changeux J.P., Chemical signaling in the brain, *Sci. Am.* 269 (1993) 58–62.
- [5] Dennis M., Giraudat J., Kotzyba-Hibert F., Goeldner M., Hirth C., Chang J.-Y., Lazure C., Chretien M., Changeux J.-P., Amino acids of the *Torpedo marmorata* acetylcholine receptor α -subunit labeled by a photoaffinity ligand for the acetylcholine receptor binding site, *Biochemistry* 27 (1988) 2346–2357.
- [6] Galzi J.L., Revah F., Black D., Goeldner M., Hirth C., Changeux J.-P., Identification of a novel amino acid α -Tyrosine 93 within the cholinergic ligand binding site of the acetylcholine receptor by photoaffinity labeling: Additional evidence for a three-loop model of the acetylcholine binding site, *J. Biol. Chem.* 265 (1990) 10430–10437.
- [7] Harel M., Kleywegt G.J., Ravelli R.B.G., Silman I., Sussman J.L., Crystal structure of an acetylcholinesterase fasciculin complex: interaction of a three fingered toxin from snake venom with its target, *Structure* 3 (1995) 1355–1366.
- [8] Karlin A., Akabas M., Toward a structural basis for the function of nicotinic acetylcholine receptors and their cousins, *Neuron* 15 (1995) 1231–1244.
- [9] Kreienkamp H.J., Maeda R.K., Sine S.M., Taylor P., Inter-subunit contacts governing assembly of the mammalian nicotinic acetylcholine receptor, *Neuron* 14 (1995) 635–644.
- [10] Kreienkamp H.-J., Sine S.M., Maeda R.K., Taylor P., Glycosylation sites selectively interfere with α -toxin binding to the nicotinic acetylcholine receptor, *J. Biol. Chem.* 269 (1994) 8108–8114.
- [11] Lee C.Y., Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their modes of neuromuscular blocking action, *Arch. Int. Pharmacodyn. Ther.* 144 (1963) 241–257.
- [12] O'Leary M.E., White M.M., Mutational analysis of ligand-induced activation of the *Torpedo* acetylcholine receptor, *J. Biol. Chem.* 267 (1992) 8360–8365.
- [13] Oswald R.E., Changeux J.P., Cross-linking of α -bungarotoxin to the acetylcholine receptor from *Torpedo marmorata* by ultra violet-light irradiation, *FEBS Lett.* 139 (1982) 225–229.
- [14] Sine S.M., Quiram P., Papanikolaou F., Kreienkamp H.-J., Taylor P., Conserved tyrosines in the α subunit of the nicotinic acetylcholine receptor stabilize quaternary ammonium groups of agonists and curariform antagonists, *J. Biol. Chem.* 269 (1994) 8808–8816.
- [15] Sine S.M., Kreienkamp H.-J., Bren N., Maeda R., Taylor P., Molecular dissection of subunit interfaces in the acetylcholine receptor. Identification of determinants of α -conotoxin M1 selectivity, *Neuron* 15 (1995) 205–211.
- [16] Sugiyama N., Boyd A.E., Taylor P., Anionic residues in the α -subunit of the nicotinic acetylcholine receptor contributing to subunit assembly and ligand binding, *J. Biol. Chem.* 271 (1996) 26575–26581.
- [17] Sugiyama N., Marchot P., Kawanishi C., Osaka H., Molles B., Sine S.M., Taylor P., Residues at the subunit interfaces of the nicotinic acetylcholine receptor that contribute to α -conotoxin M1 binding, *Mol. Pharmacol.* 52 (1998) 787–794.
- [18] Tomaselli G.F., McLaughlin J.T., Jurman M.E., Hawrot E., Yellen G., Mutations affecting agonist sensitivity of the nicotinic acetylcholine receptor, *Biophys. J.* 60 (1991) 721–727.
- [19] Tremeau O., Lemaire C., Drevet P., Pikasfeld S., Ducancel F., Boulain J.-C., Ménez A., Genetic engineering of snake toxins. The functional site of Erabutoxin a, as delineated by site-directed mutagenesis, *J. Biol. Chem.* 270 (1995) 9362–9369.
- [20] Tsai M.C., Hsieh W.H., Smith L.A., Lee C.Y., Effects of waglerin 1 on neuromuscular transmission of mouse nerve-muscle preparations, *Toxicon* 33 (1995) 363–371.
- [21] Tsigelny I., Sugiyama N., Sine S.M., Taylor P., A model of the nicotinic receptor extracellular domain based on sequence identity and residue location, *Biophys. J.* 73 (1997) 52–66.

The role of subunit interfaces for the nicotinic acetylcholine receptor's allosterism

Ferdinand Hucho^a, Christoph Methfessel^b, Anke Watty^a

^a*Freie Universität Berlin, Institut für Biochemie, Thielallee 63, D-14195 Berlin, Germany*

^b*Zentrale Forschung, Abteilung Biophysik, Bayer AG, D-51368 Leverkusen, Germany*

Abstract — Cross-linking of nicotinic acetylcholine receptors, combined with binding studies and patch-clamp electrophysiology, has proven the existence of a 'pre-existing equilibrium' of functional states and the functional role of subunit interfaces, two key postulates of the allosteric model. (©Elsevier, Paris)

Résumé — Le rôle des interfaces entre les sous-unités du récepteur cholinergique nicotinique. Des expériences de cross-linking des sous-unités du récepteur cholinergique nicotinique associées à des études de liaison et électrophysiologiques (patch-clamp) ont démontré l'existence d'un équilibre pré-existant entre plusieurs états fonctionnels et un rôle fonctionnel des interfaces entre les sous-unités, deux postulats clés du modèle allostérique. (©Elsevier, Paris)

acetylcholine receptor / allosterism / quaternary structure / cross-linking

1. Introduction

Neurotransmitter receptors, especially the ligand-gated ion channels are allosteric proteins. Allosterism, a concept developed to explain regulatory properties of proteins [1], includes several fundamental features which must be proven to make a protein an 'allosteric protein'. The most important of these are the following: i) the existence of a ligand-binding site sterically separated from the active site, which influences through conformational changes properties of the active site; ii) the existence of at least two functional states, active and inactive, in a dynamic equilibrium ('preformed', i.e., the equilibrium is present already in the absence of ligands), which is affected by ligand binding; iii) allosteric proteins are symmetrical complexes of at least two subunits, undergoing all-or-none conformational changes preserving the symmetry of the quaternary structure; and iv) ligand binding is cooperative (homotropic or heterotropic). The degree of cooperativity is expressed in terms of the Hill coefficient.

The nicotinic acetylcholine receptor (AChR) according to these criteria is an allosteric protein [2], although an 'unconventional' one [3]. Sterically separate ligand binding sites affecting each other, a dynamic equilibrium between states affected by ligand binding, a (not quite symmetrical) quaternary structure, cooperativity of ligand binding and activation (channel opening), are all well documented. The structural basis of the receptor's allosterism is beginning to emerge. A key role presumably is played by the subunit interfaces: the ion channel to be opened by cooperative agonist binding is formed by the

helices M2 contributed by the five subunits [4, 5]; and agonist binding itself is cooperative. With the agonist binding sites located on the two α -subunits which have been shown to be separated in the circular quaternary structure by the γ -subunit, communication between sites (and with the channel-forming domains) has to be 'long-range' across subunit boundaries.

Evidence has been accumulated that the agonist and competitive antagonist-binding sites are located at the interfaces between the α -subunits and its neighboring γ - and δ -subunits, respectively (reviewed in [6]). Here we show that mobility at subunit interfaces indeed is essential for allosteric action of the AChR.

2. Materials and methods

AChR was prepared from the electric organ of *Torpedo californica*. Cross-linking of receptor-rich membranes with dimethylsuberimidate (DMS) and all other chemical reactions were performed as described in [7]. Binding assays with tritiated acetylcholine were performed by ultracentrifugation after the acetylcholinesterase had been blocked by incubation with eserine [7]. Patch clamp electrophysiology with the whole-cell configuration was performed with rat muscle AChR expressed in *Xenopus* oocytes [7].

3. Results

Membrane-bound AChR was cross-linked with DMS. Binding studies showed (figure 1) that this re-

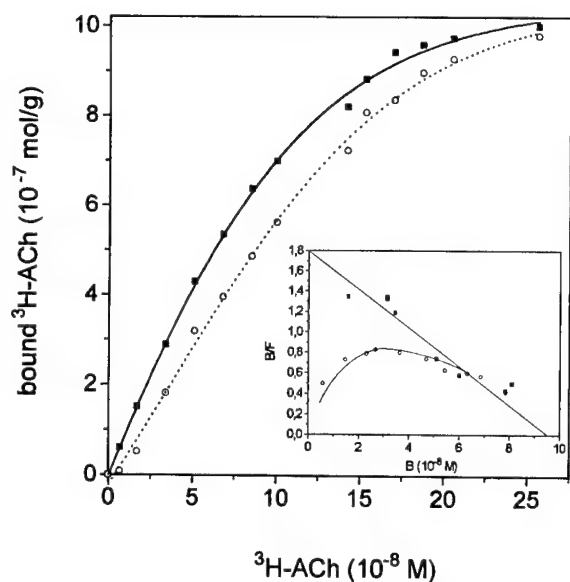


Figure 1. Properties of the nAChR from *Torpedo californica* after cross-linking with DMS. [^3H] ACh binding of native receptor (\circ) and after cross-linking with DMS (\blacksquare). Inset. Scatchard plot (B , bound [^3H] ACh; F , free [^3H] ACh). The linear Scatchard plot after cross-linking indicates the loss of cooperativity ($K_d = 50$ nM; Hill coefficient $n_H = 1.0$).

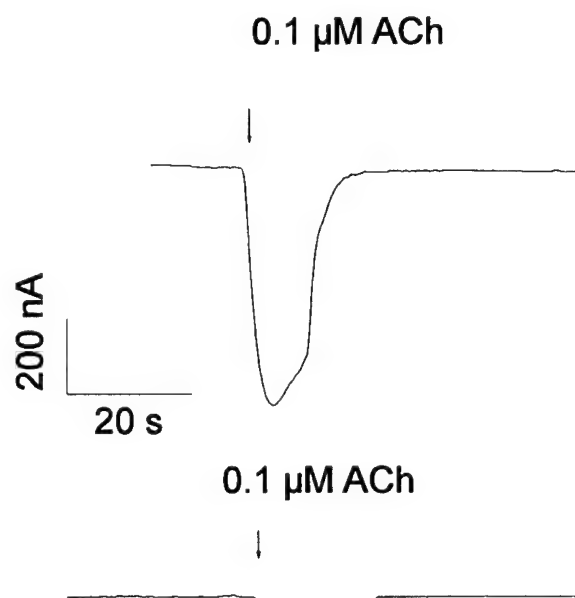
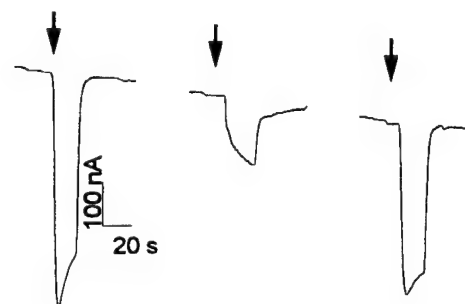


Figure 2. Effect of DMS cross-linking on ACh-induced current at nAChRs from rat muscle expressed in *Xenopus* oocytes. Whole-cell current induced by ACh application before (upper trace) and after (lower trace) 30 min of DMS cross-linking (<10% remaining current). The oocytes were washed for at least 10 min after cross-linking, to remove excess of free cross-linker.

sulted in high affinity binding ($K_D = 50$ nM). Binding was non-cooperative ($n = 1$, as compared to $n = 1.6$ for the native control). High affinity in combination with a closed channel are the characteristics of the desensitized receptor [3]. Whole-cell current measurements with cross-linked rat muscle AChR expressed in *Xenopus* oocytes showed indeed that cross-linking had fixed the receptor in a channel-closed state (figure 2). This poses the question: why was the AChR in the absence of an agonist shifted to an apparently desen-

0.1 μM ACh 1.8 mM DMS 0.1 μM ACh



0.1 μM ACh 2 mM MAI 0.1 μM ACh

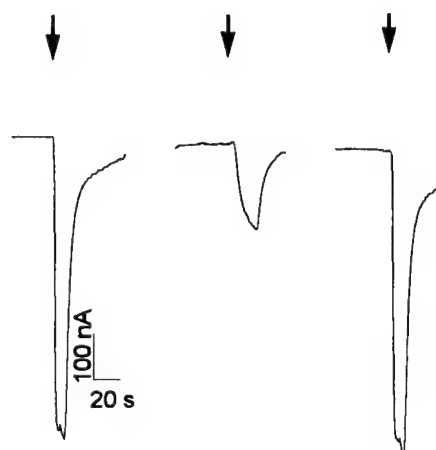


Figure 3. Agonistic effects of the imidates DMS and MAI with rat muscle nAChR expressed in *Xenopus* oocytes. Whole-cell current (rat muscle nAChR) induced by DMS application (upper trace) and by MAI application (lower trace). Whole-cell current induced by ACh application is shown before and after DMS/MAI application.

sitized state? The answer became obvious by appropriate electrophysiological measurements. DMS itself, perhaps through its positively charged imidate groups, acts as a weak agonist (figure 3).

The fixation in a desensitized state is due to bifunctional covalent cross-linking and not to monofunctional blockage of a functional group essential for desensitization. This was shown by using methylacetoimidate (MAI), the monofunctional homologue of DMS. This reagent is also an agonist, with about the same potency as DMS, but it was not able to irreversibly fix the receptor in the high affinity non-cooperative, channel closed state (figure 3). The imidate's hydrolysis products had no effect on the AChR.

If the hypothesis were right that a positively charged bifunctional cross-linking agent acts as an agonist and fixes the receptor in a desensitized state,

other bifunctional reagents without this charge should fix other states. Indeed, we observed that glutardialdehyde fixed a low-affinity, channel-closed, non-cooperative state (figure 4). But incubation with the agonist carbamoylcholine at desensitizing concentrations prior to cross-linking with glutardialdehyde resulted in irreversible fixation of the desensitized state (data not shown).

More detailed analysis of the ^3H -acetylcholine binding curve of the receptor cross-linked with glutardialdehyde in the absence of any agonist showed an interesting phenomenon (figure 4). The binding curve was biphasic; besides the low affinity part ($K_D = 5 \mu\text{M}$) Scatchard analysis indicated that about 12% of the binding sites possess a high affinity ($K_D = 35 \text{ nM}$). Obviously, the receptor in the absence of cholinergic ligands contains AChR in dif-

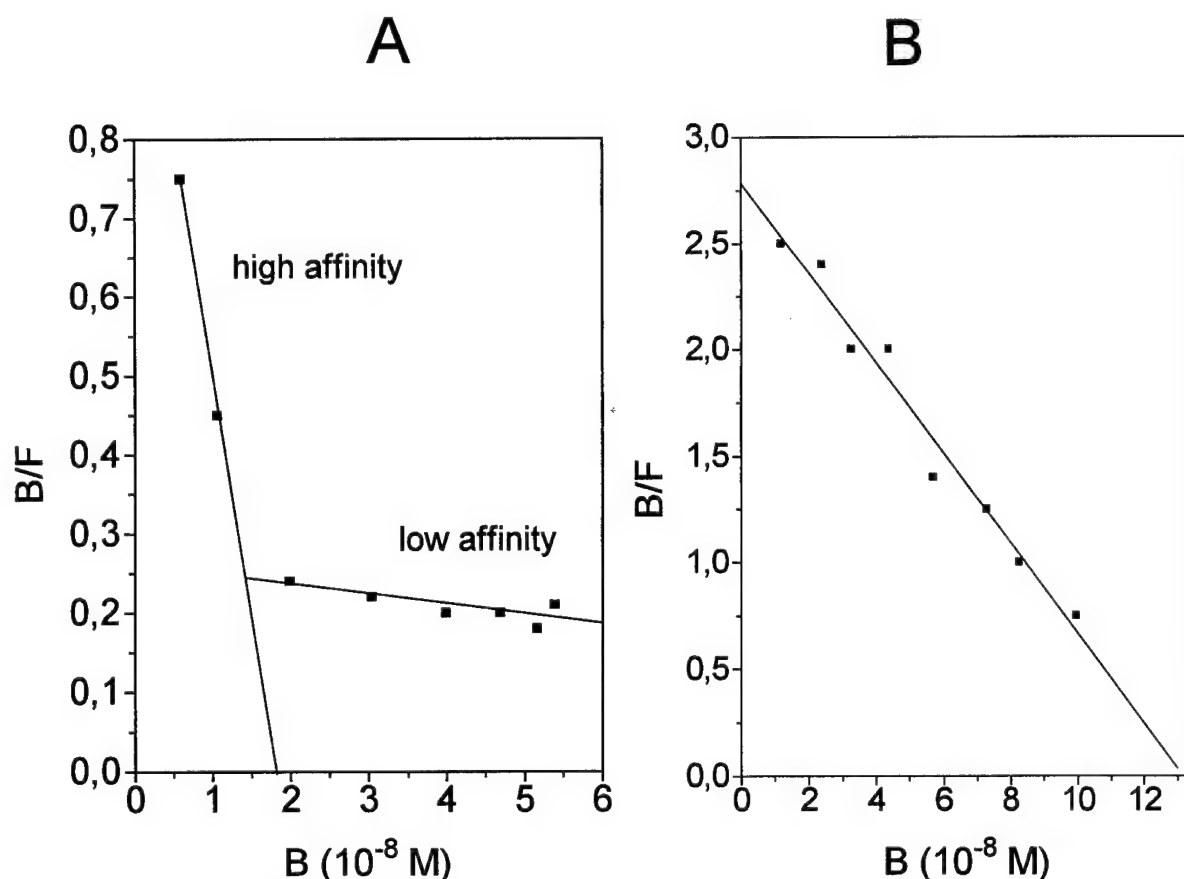


Figure 4. Properties of the nAChR from *Torpedo californica* after cross-linking with the non-agonistic cross-linker glutardialdehyde. **A.** Scatchard plot after cross-linking with glutardialdehyde in the absence of carbamoylcholine. A low-affinity binding site ($K_d = -1/m = 2-5 \mu\text{M}$; $n_H = 1.0$) and a high-affinity binding site can be seen. Inset. Scatchard plot of the high-affinity binding site alone after glutardialdehyde cross-linking, as measured by using a much higher amount of protein to achieve higher concentrations of bound ^3H ACH ($K_d = 35 \text{ nM}$; $n_H = 1.0$). The low-affinity binding site can not be seen under these conditions. **B.** Scatchard plot after cross-linking with glutardialdehyde in the presence of carbamoylcholine ($K_d = \text{nM}$; $n_H = 1.0$)

ferent functional states, which we tentatively interpret as resting and desensitized. If this interpretation is correct, we have here a direct proof for the preformed equilibrium, postulated by the allosteric theory. Because of the slowness of the cross-linking reaction the intermediate active (channel-open) state can not be fixed by this method.

4. Conclusion

Immobilization of the AChR's subunits by covalent cross-linking with bifunctional reagents abolishes allosteric properties. From our experiments we conclude that the quaternary structure and especially the mobility at the subunit interfaces is essential for the regulatory properties of the receptor. Covalent fixation of receptor states may be a useful tool for quantifying the components of an equilibrium. Shifting the equilibrium to and fixing it in a single state might also reduce the heterogeneity of a receptor preparation and facilitate certain experiments as for example attempts at receptor crystallization.

Acknowledgments

Excellent technical assistance, especially with the receptor preparations, from Giampiero Bandini is gratefully acknow-

ledged. This work was supported by the Deutsche Forschungsgemeinschaft (Sfb 312) and the Fonds der Chemischen Industrie.

References

- [1] Monod J., Wyman J., Changeux J.-P., On the nature of allosteric transitions, *J. Mol. Biol.* 12 (1965) 88–118.
- [2] Changeux J.-P., The acetylcholine receptor: an 'allosteric' protein, *Harvey Lectu.* 75 (1981) 85–254.
- [3] Galzi J.-L., Changeux J.-P., Neurotransmitter-gated ion channels as unconventional allosteric proteins, *Curr. Opin. Struct. Biol.* 4 (1994) 554–565.
- [4] Hucho F., Oberthür W., Lottspeich F., The ion channel of the nicotinic acetylcholine receptor is formed by the homologous helices M II of the receptor subunits, *FEBS Lett.* 205 (1986) 137–142.
- [5] Giraudat J., Dennis M., Heidmann T., Haumont P.-Y., Lederer F., Changeux J.-P., Structure of the high-affinity binding site for noncompetitive blockers of the acetylcholine receptor: [³H] chlorpromazine labels homologous residues in the beta and delta chains, *Biochemistry* 26 (1987) 2410–2418.
- [6] Hucho F., Tsetlin V., Machold J., The emerging three-dimensional structure of a receptor. The nicotinic acetylcholine receptor. Review, *Eur. J. Biochem.* 239 (1996) 539–557.
- [7] Watty Anke, Methfessel C., Hucho F., Fixation of allosteric states of the nicotinic acetylcholine receptor by covalent cross-linking, *Proc. Natl. Acad. Sci. USA* 94 (1997) 8202–8207.

Allosteric modulation of neuronal nicotinic acetylcholine receptors

Bruno Buisson, Daniel Bertrand

Department of Physiology, Medical Faculty, 1, rue Michel Servet, CH-1211 Geneva 4, Switzerland

Abstract — The structure-function relationship of the neuronal nicotinic acetylcholine receptor is examined in the light of the allosteric concepts. Effects of site-directed mutagenesis as well as those caused by allosteric effector of the physiological and pharmacological receptor properties are discussed. (©Elsevier, Paris)

Résumé — **Modulation allostérique des récepteurs nicotiniques neuronaux.** La relation entre la structure et la fonction du récepteur nicotinique neuronal à l'acétylcholine est examinée à la lumière des concepts allostériques. Les effets de mutations dirigées ainsi que ceux causés par des effecteurs allostériques sur les propriétés physiologiques et pharmacologiques du récepteur sont examinées en fonction de ces concepts. (©Elsevier, Paris)

nicotinic acetylcholine receptors / allosteric concepts / site-directed mutagenesis

1. Introduction

The discovery of the nervous electrical activity and physiological description of the action potential [50] led to rapid progress in studies of the nervous system. It was soon realized, however, that neuronal firing represents only one of the steps in the elucidation of the nervous system activity but that comprehension of signal transmission across neurons was another major breakthrough required to approach the understanding of brain function. Transmission of electrical activity in the mammalian brain almost exclusively relies on the chemical synapses. Propagation of the action potential at the synaptic bouton causes the release of neurotransmitter, which passively diffuses across the synaptic cleft and binds to integral proteins inserted in the postsynaptic membrane. Regeneration of an electrical signal by the neurotransmitter molecules depends upon the opening of ions permeable channels. Thus, along the evolution, some highly specialized proteins, expressed in the postsynaptic membrane, have developed a dual function with the capacity of ligand binding and the formation of an aqueous ionic pore. These molecules are identified as ligand-gated channels (LGCs). Neuronal nicotinic acetylcholine receptors (nAChRs) belong to a broad family of neuroreceptors that includes GABA_A, glycine and serotonergic 5-HT₃ receptors [38]. They have been demonstrated to be activated within microseconds [20, 33] by the presence of acetylcholine (ACh) at micromolar concentrations. Cloning and sequencing of the neuromuscular junction nAChR subunits [81] represented an important step in the understanding of their structure. Derived from these studies it was

proposed that a single receptor complex is made by the assembly of five subunits, each of which spans four times the membrane. These proteins, which are inserted into the cytoplasmic membrane, form at the same time the ligand binding site, in the extracellular domain, and the ionic pore, aligned with the pseudosymmetrical axis of the pentamer [14]. Library screening using low stringency hybridization with partial sequences of the DNA coding for the neuromuscular nAChR led to the identification of several related genes coding for the neuronal nAChRs. Up to now a total of 11 closely related cDNAs coding for neuronal nAChRs proteins have been identified in vertebrates [62, 67].

Neuronal nAChRs can be divided in two main groups depending upon their sensitivity to the snake toxin α -bungarotoxin (α -Bgt) [16]. Sensitivity to α -Bgt was employed for a long time as criteria to identify neuronal nAChRs from muscular receptors, which are blocked by this competitive inhibitor in the nanomolar range. In contrast, neuronal nAChRs, such as ganglionic receptors, were shown to be insensitive to this toxin even for concentration a 1000-fold higher [1]. With the identification of a group of α subunits ($\alpha 7$ - $\alpha 9$) that can reconstitute functional nAChRs when expressed alone in *Xenopus* oocytes or in cell lines it was also possible to demonstrate that this class of receptors is inhibited by α -Bgt [24, 32, 43, 84, 87, 100]. Several studies have further illustrated that these initial findings could take into account the important α -Bgt labeling observed in the central nervous system [17, 117]. Given their capacity to reconstitute homomeric receptor, $\alpha 7$ subunits were employed as a model in many structure function relationship studies. The main advantage provi-

ded by this receptor is that engineering of a single subunit is sufficient to alter the entire receptor complex (reviewed in [6]).

Rapid progresses in molecular biology and the introduction of reconstitution techniques (reviewed in [7, 11]) allowed to investigate physiological and pharmacological properties of neuronal nAChRs in isolation. Combination of site-directed point mutagenesis and electrophysiological recordings further increased the possibilities of studies of these LGCs at the molecular level. The purpose of this work is to review, in the light of the allosteric model, the properties of the neuronal nAChRs and to place them in perspective of both our current knowledge of the wider family of neuroreceptors as well as related neurological disorders.

2. The nAChR: a model of allosteric protein

High resolution electron micrograph studies, at 9 Å resolution, of the *Torpedo* electric organ realized by Unwin [110] confirmed the hypothesized protein structure of the neuromuscular junction receptor [27] and is used as a tridimensional model for other receptors comprising the same protein characteristics (reviewed in [5, 38]). Schematic organization of a single receptor complex, based on these studies, is presented in *figure 1A*.

Since nAChRs result from the assembly site of five subunits, they constitute an excellent prototype of allosteric proteins in which the ligand binding is distinct from the 'reaction site', that is, the ionic pore. Allosteric proteins, as firstly proposed by Monod, Wyman and Changeux (MWC), have the particularity to undergo spontaneous transitions between different conformations, some of which being preferentially stabilized by the presence of ligand(s). Such transitions are possible when assuming a 'concerted' rearrangement of the protein subunits (i.e., the different subunits change their conformation in cooperativity). Although initially formulated for cytosolic proteins, such as hemoglobin [28], the allosteric theory can be extended to several types of molecules, ranging from DNA to LGCs [38, 63, 96]. In the case of the nAChRs, it is assumed that binding of the natural ligand acetylcholine (ACh) stabilizes the receptor in the active open state. To take into account the channel closure observed upon prolonged exposure to high agonist concentrations, a three-state model, presented in *figure 1B*, represents the minimal number of states required for the description of the channel properties [48]. This phenomenon, typically observed for LGCs, is referred as 'receptor desensitization'. According to this three-state model the receptor can take any of the three conformations,

basal (closed), active (open) or desensitized (closed). Thus, and deriving from the allosteric concept, it can be assumed that some of the nAChR 'binding sites' will display different properties according to the state which is considered.

Studies of the muscle nAChR using photoaffinity labeling [41] or other techniques [25, 55, 103] have revealed that the ligand binding site lies at the interface between an alpha and the adjacent subunit (γ or δ (reviewed in [6])). Sequence alignment of the N-terminal amino acid sequences of muscle and neuronal nAChRs unveiled the high conservation of residues identified as determinant of the ACh binding site. Mutation of these aromatic residues in the $\alpha 7$ neuronal nAChR caused an important reduction of the receptor sensitivity to ACh [36], as shown by the decrease in the EC_{50} (the concentration of agonist required to produce half activation of the receptor). These studies lead to the conclusion that in the alpha subunit the agonist binding pocket comprises at least three main loops referenced to as A, B and C (*figure 1C*). Further site-directed mutagenesis studies have confirmed that, as predicted from the muscle experiments, ACh binds at the interface between alphas and the adjacent subunits. Namely, it was observed that mutations in the so-called complementary domain, in the adjacent subunit, cause important changes in the receptor EC_{50} as well as its pharmacological profile [21]. Concomitant with these studies, other experiments conducted on the muscle nAChRs have illustrated the high degree of homology observed between nAChRs [14]. Attempts of modelisation of the N-terminal structure using a bacterial copper binding protein template [107] further support the initial schematic representation of the ACh binding site. Thus, although the exact tridimensional structure of the ACh binding pocket remains to be determined at the atomic level, all evidence suggests that the ligand is binding in the extracellular domain of the proteins at the interface between two adjacent subunits.

3. The Monod-Wyman-Changeux model

Modeling of the allosteric proteins properties in steady state conditions, as proposed by MWC, can readily be done using the following equation:

$$\bar{A} = \frac{1}{1 + L \cdot \left\{ \frac{1 + [ACh]/K_b}{1 + [ACh]/K_a} \right\}^n}$$

where \bar{A} is the fraction of receptors in the open state, L is the isomerization constant, K_a and K_b are the respective microscopic dissociation constants for

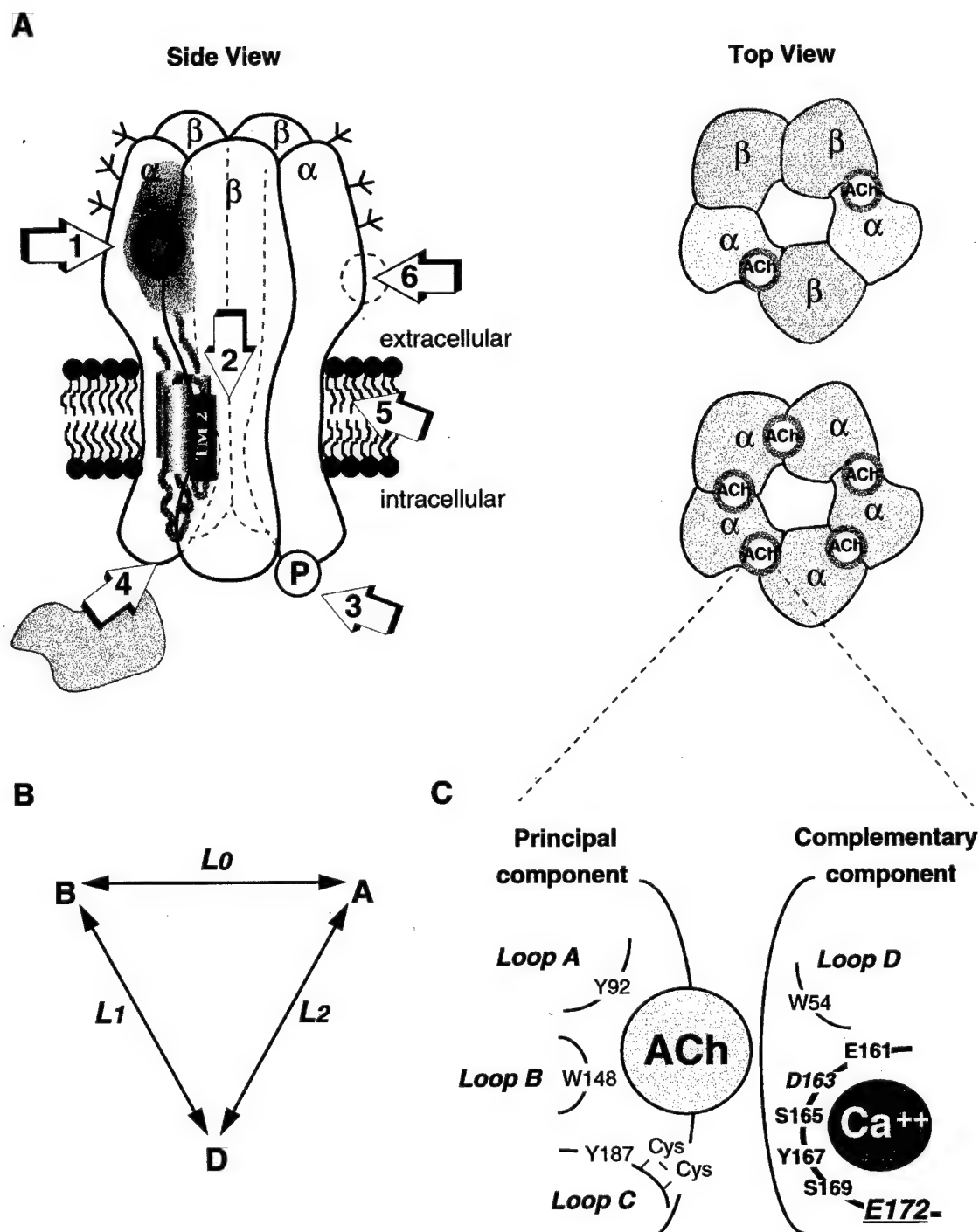


Figure 1. The neuronal nAChRs: a prototype of an allosteric protein. **A.** Schematic organization of the receptor. Transmembrane organization and possible interaction sites are represented on the side view (left panel). Arrows correspond to: 1) ligand-binding site(s); 2) ionic pore; 3) putative phosphorylation domain(s); 4) possible site(s) of interaction with cytosolic or protein(s) of the cytoskeleton; 5) phospholipid interaction site(s); and 6) extracellular allosteric binding site(s). Heteromeric and homomeric receptors with possible ACh binding sites are represented in the top view (right panel). **B.** Three-state allosteric model where: B, basal (closed) state; A, active (open) state; D, desensitized (closed) state; L_0 , L_1 , L_2 , isomerization constants. **C.** Schematic representation of the ligand binding site at the interface between an α and the adjacent subunit. Residues participating to the formation of the principal and complementary components are represented.

the agonist while $[ACh]$ is the agonist concentration. n corresponds to the number of binding sites for the agonist (ACh).

When considering the transition from the close resting state to the open conducting state, a first prediction derived from this equation is that, depending upon the value of L , the EC_{50} as well as the fraction of receptors that can be stabilized in the open conformation will vary. The lowest is the L value, the easiest is the transition. Two alternative conditions can be considered in which the L value may be modified. First, mutations of amino acid residues may cause a modification of the protein stability and thereby may affect the concerted transitions between different conformational states and, as a correlate, may change the value of the intrinsic isomerization constant (L). Second, the presence of a molecule that could bind to the protein at a site near or distant from the ACh binding pocket may affect the L coefficient, as initially described by Rubin and Changeux [95]. It is of value to recall that the Greek prefix 'allo' means 'elsewhere' because the allosteric model postulates that different regions of one molecule are cross-linked in their behavior. As we shall see, the two conditions listed above have been experimentally observed with the neuronal nAChRs.

Among the different methods that allow to investigate the properties of LGCs, the electrophysiological approach offers the main advantage of a real time monitoring, with high time resolution, of the almost synchronous behavior of up to 1000 of proteins expressed in a cell membrane. Using the so-called 'voltage clamp' methods, it is possible to follow the net and macroscopic ion flux evoked by the opening of many LGCs while clamping the membrane potential at the same time. Ultimate resolution is reached, with the patch clamp technique, by recording small patches of membrane where the opening of a single channel can be observed. In optimal conditions, the transitions of a single LGC molecule can be followed with a pico-Amper and microsecond resolution [7, 11, 20]. In the case of the neuronal nAChRs, investigations at the single channel level are limited by an important run down, that is, the 'loss' of channel activity (in outside out patches) even in the absence of agonist [9, 79, 85].

4. Electrophysiological recordings: a probe for the allosteric model

Site-directed mutagenesis of a single amino acid comprised within the second transmembrane segment (TM2) of $\alpha 7$, with the replacement of a leucine in threonine (L247T), was found to produce pleiotropic alterations of the receptor properties [4, 91].

Most important modifications being a 200-fold increase in the agonist sensitivity, loss of desensitization, transformation of the receptor pharmacological profile and presence of a new conductance at the single channel level (reviewed in [6]). Interestingly, all these modifications could be taken into account assuming that the L247T mutation renders conductors a desensitized state of the nAChR.

When analyzed on the basis of the allosteric model, effects of the L247T mutation can be described assuming a new conductive state and a low isomerization constant [40]. A further prediction of the allosteric model is that for a low L value a fraction of the receptor corresponding to $1/L$ should be open even in absence of agonist. Although this fraction is close to zero for L values as large as 10^6 - 10^8 needed for the modeling of the wild type (WT) $\alpha 7$ receptor this fraction becomes non-negligible when a L coefficient as low as 20 is employed for the description of the L247T mutant. Detailed analysis of the properties of oocytes expressing the L247T mutant revealed that a constant current must be injected to hold at -100 mV cells expressing this nAChR. This leakage current being several fold larger than that needed for non-injected oocytes [8]. Furthermore, it was found that this current was reduced by incubation of the cell in presence of the competitive inhibitor methyllycaconitine (MLA; [82]). From these and other observations it was concluded that, as predicted by the allosteric model, a fraction of receptors are spontaneously open in cells expressing the L247T mutant [8].

Another important finding obtained from the analysis of the L247T mutant concerns the modification of the pharmacological profile of the receptor. While the WT $\alpha 7$ nAChR is inhibited in a competitive manner by dihydro- β -erythroidine (DH β E), this compound is a full agonist at the L247T mutant. Although at first surprising, these data are readily explained assuming that in the WT receptor DH β E stabilizes a desensitized state which becomes conductor in the L247T mutant. In contrast to DH β E the other competitive antagonist MLA does not evoke large amplitude currents when applied to the L247T but, as described above, closes the channels. The differential effect of these two competitive antagonists can be explained by assuming that they may stabilize different conformational states, namely the 'closed resting state' for MLA and one of the 'desensitized state' for DH β E. Further support for this hypothesis is provided by the observation that α -Bgt behaves like MLA, either on the $\alpha 7$ WT or on the L247T mutant receptor. At WT $\alpha 7$ nAChRs, MLA and DH β E are identified as 'competitive antagonists'. At the L247T mutant, MLA and α -Bgt may be classified as 'inverse agonists' whereas

DH β E is a full agonist. Thus, the allosteric interpretation of the $\alpha 7$ nAChR mutants points, once again, to the semantic limits of the current pharmacological nomenclature.

Simultaneous introduction of two mutations in TM2 (L247T and V251T) causes a further alteration of the receptor properties. Oocytes expressing this type of receptor display very large leak currents, in the μ A range, that are reduced by applications of low ACh concentrations [8]. As confirmed by measurements of the cell-input resistance, this weak current is attributable to spontaneous opening of an important fraction of the receptors. Furthermore, current-voltage relationships (I-V) recorded in the absence or presence of agonist revealed that spontaneously open channels display a reversal potential similar to that of the $\alpha 7$ WT nAChR. Increasing the agonist concentration results first in smaller reduction of the channel closure and then in an inward current. This complex channel behavior can be explained by the allosteric model assuming that spontaneous opening reflects the fraction of receptor which is in the desensitized, high affinity, D state while current activation by high agonist concentrations results from the stabilization of the receptor in the active and low affinity A state.

As described above, another important prediction of the allosteric model is that substances that can bind to the protein at a site distinct from that of the native ligand binding pocket may also affect the overall state equilibrium [95]. Compounds that increase the apparent isomerization L coefficient are called negative allosteric effectors whereas substances that reduce the apparent L coefficient are called positive allosteric effectors. Application of a negative allosteric effector should result in a reduction of the protein activation while a positive effector would reinforce the agonist effect.

Electrophysiological studies of native nAChRs had shown that these receptors are potentiated, at physiological concentration, by extracellular calcium [80, 114]. A high calcium concentration (i.e., > 2 mM) induces both a decrease of the single channel conductance and an increase of the mean open time. At the macroscopic level, an increase of the ACh-evoked current is observed. In the view of these results it was suggested that calcium acts as a positive allosteric effector on the neuronal nAChRs where it could bind with an affinity in the millimolar range [65]. Based on putative sequence homology with natural calcium binding proteins, such as calmodulin, site-directed mutagenesis experiments conjugated with functional studies were designed. Results obtained with this approach confirmed the existence in the extracellular domain of the $\alpha 7$ nAChR of a consensus calcium binding motive, the mutation of

which suppresses calcium potentiation [37]. Moreover, introduction of this consensus sequence in the extracellular N-terminal part of the 5-HT $_3$ receptor, that is natively insensitive to the extracellular calcium concentration, was sufficient to cause calcium potentiation [37].

Other studies had previously pointed out that steroids, and more specifically progesterone, are negative allosteric effectors at the major brain $\alpha 4\beta 2$ nAChR [3, 112]. Although these studies were conducted on the chick nAChRs it was recently shown that progesterone also behaves as a negative allosteric effector on human nAChRs reconstituted either with the $\alpha 4\beta 2$, $\alpha 3\beta 2$ or $\alpha 7$ subunits (Buisson et al., submitted). In agreement with these observations, dose-responses to ACh recorded in control conditions or in presence of progesterone are readily fitted with an allosteric equation assuming that progesterone reduces the apparent isomerization coefficient L_0 of the $B \leftrightarrow A$ equilibrium. Nevertheless, since progesterone increases the nAChRs desensitization without affecting the receptor apparent affinity for ACh, it is likely that this steroid may stabilize one the nAChRs desensitized states with a concomitant reduction of the isomerization constant L_2 (figure 1B). Of more interest is the observation that another 'female' steroid, 17 β -oestradiol, potentiates human $\alpha 4\beta 2$ nAChRs while it inhibits $\alpha 3\beta 2$ and homomeric $\alpha 7$ nAChRs (Buisson et al., submitted). The 17 β -oestradiol potentiation of $\alpha 4\beta 2$ nAChRs occurs with no change in the receptors apparent affinity for ACh and of the desensitization profile. Thus, 17 β -oestradiol may preferentially stabilize the A state. In favor of this hypothesis is the observation that the fit of the 17 β -oestradiol dose-response profile with the allosteric equation [95] yields an isomerization constant of 60 for the $B \leftrightarrow A$ equilibrium, whereas it yields $L_0 = 220$ in control conditions. With an identical $\beta 2$ subunit, 17 β -oestradiol mediates two opposite allosteric modulations that are related to the presence of one α subunit: $\alpha 4$ for the potentiation and $\alpha 3$ for the inhibition. At human $\alpha 4\beta 2$ nAChRs 17 β -oestradiol is a positive allosteric modulator whereas Ca^{2+} (at concentrations > 2 mM) is a negative allosteric modulator [9] (Buisson et al., submitted). However, the same compounds behave in opposite directions at human $\alpha 3\beta 2$ nAChRs where 17 β -oestradiol is a negative allosteric modulator and Ca^{2+} a positive one [59]. Although performed with reconstituted receptors, these experiments raise the notion that according to their subunit composition as well as to their cellular location (post- versus pre-synaptic), modulation of neuronal nAChRs could have opposite effects on the synaptic transmission mechanisms in a given physiological background. This illustrates that, as previously suspected, the

composition and the distribution of the nAChRs subunits around the axis of pseudo-symmetry is determinant for the interaction of allosteric modulators [6].

More recently it was shown that the anthelmint ivermectin (IVM) is a powerful allosteric potentiator at both chick and human homomeric $\alpha 7$ nAChR [58]. The direct mode of action of IVM on the $\alpha 7$ receptor was supported by the observation that application of this drug does not modify the reversal potential of the current-voltage relationship indicating that increase in the ACh-evoked responses cannot be attributed to the activation of another ionic channel. In addition, an important potentiation was also observed with the calcium impermeant mutant E237A [4], indicating that the IVM effects are independent of the calcium influx.

Taken together these data confirm the existence of negative as well as positive allosteric effectors on the neuronal nAChRs and support the hypothesis that these integral membrane proteins can take several conformations, some of which being preferentially stabilized by the ligand or compounds that can bind in the ligand-binding pocket. In this respect it is of value to note that ligands are typically classified in three categories, depending on their physiological effects. The first class being the natural agonist, which is thought to fully activate the receptor. The second class corresponds to the competitive inhibitors, which are thought to stabilize the receptor in an inactive configuration. The third class are the partial agonists, which can only evoke a fraction of the activation caused by the natural agonist. Several studies, as in the case of the glutamate receptor [71], have revealed that, when applied concomitantly to the receptor natural ligand, partial agonists provoke an inhibition. Although at first surprising, this observation can be taken into account assuming that, given their chemical nature, partial agonists can stabilize only a fraction of the receptor in the active state. It can therefore be concluded that, in term of allosteric model, partial versus full agonist effects should depend upon the isomerization coefficient L . As a consequence it can be proposed that exposure to a positive allosteric effector that reduces the apparent L coefficient should also cause a modification of the receptor pharmacological profile. This hypothesis that was indeed confirmed with IVM as shown by the transformation of the pharmacological profile of chick $\alpha 7$ to dimethyl-phenyl-piperazinium (DMPP). In control conditions DMPP behaves as a very weak agonist at the chick $\alpha 7$ nAChR. However, short pre-applications of IVM can 'transform' this compound as a full agonist [58].

5. Discussion

Neurotransmission in the vertebrate central nervous system (CNS) is almost exclusively mediated by synapses with their cohort of neuroreceptor molecules. LGCs with their rapidly activating ionic pore have been shown to play an important role in fast signal transmission. Neuronal nAChRs, which belong to the so-called four transmembrane LGC superfamily, are homologous of GABA_A, glycine and 5-HT₃ receptors [38]. Within this superfamily, conservation of the tridimensional protein organization as well as its functional properties are such that a switch of the ionic selectivity of $\alpha 7$ nAChR, from cation to anion, can be engineered by substituting a few amino acids that face the ionic pore for their GABA_A counterparts [39]. The conservation of the functional domains is illustrated by the production of a chimeric receptor between the homomeric $\alpha 7$ nAChR and the 5-HT₃ receptor. The chimera displays the pharmacological specificity of a nAChR but the ionic pore properties of the native 5-HT₃ [31]. Moreover, repetition of the chick $\alpha 7$ L247T mutation in the muscle nAChR [60] as well as in the GABA_A [106] and 5-HT₃ [119] receptors produces equivalent phenotypes.

An important paradigm that has to be kept in mind in modeling receptor properties is their ability to be in multiple functional states. Models that can be considered for the description of LGCs are subdivided in two basic classes with: a) induced fit reactions; and b) allosteric processes. While induced-fit models [57] assume that binding of the ligand causes a modification in the protein conformation, the allosteric scheme postulates that, in absence of ligand, the protein undergoes spontaneous conformational changes and that addition of the ligand preferentially stabilizes one or more conformational states [76]. For an allosteric analysis of its properties, a LGC must fulfill the following criteria: i) it results from the assembly of at least two protomers; ii) it possesses one or more axis of rotational symmetry; iii) it exists in, at least, two freely interconvertible conformational states; and iv) transition from one state to the other involves a 'concerted' conformational change of the protomers.

Isolation of genes coding for nAChRs and their reconstitution in host systems unveiled that indeed these receptors result from the assembly of five subunits (reviewed in [6, 38]). Moreover, an axis of pseudo-symmetry can be observed at the ionic pore level, which lies in the center of the subunit assembly [14, 110]. A clear distinction can be made between the induce-fit and allosteric models regarding the possibility that the protein spontaneously undergoes conformational changes. In the case of the al-

losteric model, spontaneous channel opening should be present, even if these occurrence may be very low, whereas induce-fit models can hardly predict such openings. In agreement with this observation spontaneous single channel activity was observed for the muscle receptor [54]. For the neuronal nAChR, site directed mutagenesis done in TM2 have further illustrated that cells expressing the $\alpha 7$ L247T mutant display a significant fraction of current even in the absence of agonist [8]. Similar mutation performed in the GABA_A receptor yielded proteins that also showed a significant fraction of opening in absence of ligand [83, 106]. Although the observation of 'open' *Torpedo* nAChRs using electronic microscopy suggests a conformational rearrangement of the transmembrane domains [111], the functional characterization of concerted transitions remains to be established. Strategies employing concatemers of subunits allowed to demonstrate the cooperative interaction of the potassium channel subunits in the gating mechanism [108]. Nevertheless, it is very likely that equivalent concerted mechanisms occur during the allosteric transitions of LGCs. Altogether, these data strongly support the hypothesis that LGCs represent typical allosteric proteins since they can undergo spontaneous transitions.

Recent studies performed with cGMP gated channels indicate that such channels also display a spontaneous activity in the absence of cGMP (the intracellular ligand) and this was also invoked as a clear demonstration of the allosteric nature of the transitions that occur during the receptor activation [96]. Spontaneous receptor activity has also been reported for G-protein coupled receptors (GPCRs) [15, 29, 42, 86, 98]. Thus, the ability of undergoing allosteric transitions seems a common property of many membrane receptor proteins.

One important feature, that has been poorly explored up to now, is the allosteric modulation of neuronal nAChRs by intracellular factors. Investigations performed with the glutamate NMDA receptor have demonstrated that intracellular proteins (such as actin filaments of the cytoskeleton) are able to modulate the receptor properties [94]. The analysis of the amino acid in the long intracellular loop comprised between transmembrane segments 3 and 4 (TM3 and TM4) reveals the presence of a number of consensus sequences for kinase phosphorylations. Preliminary works have shown that activation of the cyclic AMP (cAMP) pathway by peptidic hormones such as calcitonin gene-related peptide (CGRP) or vasoactive intestinal peptide (VIP) can modify the properties (agonist affinity and/or desensitization profile) of muscle and neuronal nAChRs, respectively [46, 69, 78]. These effects may be related to phosphorylation/dephosphorylation events following the cAMP

activation of protein-kinase A (PKA). The rat nAChR $\alpha 4$ subunit is phosphorylated by PKA [53] and PKC [30]. Moreover, the up-regulation of human $\alpha 4\beta 2$ nAChRs expressed in K177 cells involve PKA- and PKC-mediated mechanisms [44]. In vitro experiments indicate that the $\alpha 7$ intracellular loop (TM3-TM4) is a substrate for PKA [77]. Since careful investigations of phosphorylation/dephosphorylation effects on neuronal nAChRs are needed, it is reasonable to assume that, as observed for other membrane receptors, phosphorylations may modify the intrinsic properties of nAChRs (changing the L value) and thus the pharmacological profile and/or gating properties. Moreover, phosphorylations may modify the interaction of nAChRs with other intracellular factors such as Grb2 [19] or agrin [35]. In myoblasts, cytoplasmic proteins that are closely associated with the nAChRs such as agrin and rapsyn have been characterized for targeting and/or stabilizing nAChR molecules in postsynaptic regions [13, 90, 121]. Gephyrin has recently been implicated in the clustering of glycine receptors in brain neurons [56] while proteins of the PDZ family play an identical role at ionotropic glutamate receptors [26]. The possible influence of these docking molecules on the receptor functions awaits a further characterization, since in many studies, recordings of the nAChRs are performed with the presence of endogenous factors that are hardly controlled. Studies performed with GPCRs indicate that their pharmacology is influenced by the association-dissociation with trimeric G-proteins. Most of the time, GPCRs have a lower affinity for their natural agonist when they are associated to 'inactive' G-proteins [92, 116]. Moreover, the overexpression of G-proteins is able to stabilize GPCRs in their active state [12]. Thus, similar interactions may be postulated between neuronal nAChRs and intracellular proteins that remain, however, to be identified.

Investigations performed either with animals or with freshly isolated slices/neurons pointed out that many physiological and/or synthetic compounds could modify the LGCs properties and therefore, the synaptic transmission efficacy. Among these molecules, the benzodiazepines (BZDs) represent the prototype of allosteric effectors. Identified for their anti-convulsant and anxiolytic properties, the binding site of these compounds has been located on the GABA_A receptor but at a site that is distinct from the agonist one [101], suggesting that, as observed a few years ago for the enzymes (reviewed in [28]), complex receptor proteins can present alternative binding sites for allosteric effectors. Moreover, the sensitivity toward BZDs depends on the receptor subunits: as an example, GABA_A receptors containing the $\alpha 4$ subunit are less sensitive to BZDs [102, 115].

The discovery that steroid binds and modulates GABA_A receptors further confirmed the idea that LGCs could be negatively and positively regulated by different allosteric molecules belonging to a same family [47, 68]. As observed for the BZDs, a steroid- and subunit-specific modulation was observed in experiments performed with reconstituted receptors [61, 89]. The allosteric modulation by steroids is a feature that is shared by many LGCs since these cholesterol-derived compounds are still active at glutamate NMDA receptors [72] and also at neuronal nAChRs (see above). Thus, steroids originating either from the gonads and/or de novo synthesized in the brain [2] can target and modulate different types of LGCs. The potential interest of using such compounds for the treatment of neuropathologies [97] is emphasized by the empirical, but very promising results obtained with the estrogen replacement therapy (ERT) for people suffering from Alzheimer's disease [49].

Immunocytochemistry as well as in situ hybridization studies have revealed that neuronal nAChR subunits are broadly expressed in the fetal and adult brain [74, 93] and that, most of the time, a given region expressed more than three different subunits [22, 23, 51, 52, 88, 93, 113]. From a theoretical point of view, the nAChRs composition and subunit rearrangement could be relatively complex [6] as well as their cellular location (pre- versus post-synaptic in neurons). Moreover, all nAChRs are, to some degree, permeable to Ca²⁺ with the highest permeability observed for the homomeric $\alpha 7$ nAChRs [4, 100]. Thus, and according to the pivotal role of calcium in synaptic transmission/plasticity, neuronal nAChRs may constitute determinant actors/modulators of the synaptic transmission [93, 118]. Electrophysiological investigations performed with rodent brain tissues have indicated that presynaptic nAChRs containing the $\alpha 7$ subunits control the release of glutamate [45, 73]. However, $\alpha 7$ -containing nAChRs have been identified both in post and presynaptic membranes at chick ciliary ganglion synapses [18, 52] where they mediate up to 90% of the fast cholinergic transmission [109, 122]. Thus, α -Bgt sensitive nAChRs can be present on both sides of synaptic cleft. Non- $\alpha 7$ nAChRs subunits have been implicated in the release of other neurotransmitters such as GABA [64, 66, 75, 120], dopamine [70], serotonin [99] and also ACh [18, 34]. Thus, the distribution and the roles of nAChRs follow a complex scheme. With the exception of a few compounds (which can be counted on one hand), pharmacological investigations performed on reconstituted or on native receptors did not allow the identification of agonists/competitive antagonists that are specific to one nAChR subtype. Moreover, non-competitive an-

tagonists, such as open channel blockers, can display a much larger spectrum of action than initially expected [10]. Therefore, the characterization of allosteric effectors such as 17 β -oestradiol, that are able to specifically potentiate one type of nAChR, opens alternative pharmacological pathways for the identification/design of molecules that can be target of one type of nAChR. This observation is of particular relevance when knowing that patients affected by autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) present mutations in the nAChR $\alpha 4$ subunit [104, 105]. Thus, and as recently illustrated for the GABA_A receptor [102], alteration of a single subunit can lead to a neuropathological profile. Since presynaptic boutons and postsynaptic membranes express nAChRs that could be of different compositions (see above), physiological as well as pharmacological controls of these synapses must implicate compounds highly specific for a given receptor subtype, and that allow a precise adjustment of the synaptic transmission efficacy. The identification and comparison of the 17 β -oestradiol binding sites on human $\alpha 4$ and $\alpha 3$ nAChRs subunits shall constitute a determinant step in the design of targeted allosteric modulators for the neuronal nAChRs.

Acknowledgments

We are grateful to Fabienne Picard for comments on the manuscript. Work is supported by the Swiss National Foundation No. 31-37191.93 and by the Office Fédéral de l'Éducation et des Sciences (to D.B.).

References

- [1] Ascher P., Large W.A., Rang H.P., Studies on the mechanism of action of acetylcholine antagonists on rat parasympathetic ganglion cells, *J. Physiol. (Lond.)* 295 (1979) 139–170.
- [2] Baulieu E.E., Neurosteroids: of the nervous system, by the nervous system, for the nervous system, *Rec. Prog. Horm. Res.* 52 (1997) 1–32.
- [3] Bertrand D., Valera S., Bertrand S., Ballivet M., Rungger D., Steroids inhibit nicotinic acetylcholine receptors. *Neuroreport* 2 (1991) 277–280.
- [4] Bertrand D., Revah F., Galzi J.L., Hussy N., Mulle C., Bertrand S., Ballivet M., Changeux J.P., Unconventional pharmacology of a neuronal nicotinic receptor mutated in the channel domain, *Proc. Natl. Acad. Sci. USA* 89 (1992) 1261–1265.
- [4] Bertrand D., Galzi J.L., Devillers-Thiéry A., Bertrand S., Changeux J.P., Mutations at two distinct sites within the channel domain M2 alter calcium permeability of neuronal $\alpha 7$ nicotinic receptor, *Proc. Natl. Acad. Sci. USA* 90 (1993) 6971–6975.
- [5] Bertrand D., Galzi J.L., Devillers-Thiéry A., Bertrand S., Changeux J.P., Stratification of the channel domain in neu-

- rotransmitter receptors, *Curr. Opin. Neurobiol.* 5 (1993) 688–693.
- [6] Bertrand D., Changeux J.P., Nicotinic receptor: An allosteric protein specialized for intercellular communication, *Semin. Neurosci.* 7 (1995) 75–90.
- [7] Bertrand D., Buisson B., Krause R.M., Hu H.Y., Bertrand S., Minireview: Electrophysiology: A method to investigate the functional properties of ligand-gated channels, *J. Recept. Signal. Transduct. Res.* 17 (1997) 227–242.
- [8] Bertrand S., Devillers-Thiéry A., Palma E., Buisson B., Edelstein S.J., Corringer P.J., Changeux J.P., Bertrand D., Paradoxical allosteric effects of competitive inhibitors on neuronal $\alpha 7$ nicotinic receptor mutants, *Neuroreport* 8 (1997) 3591–3596.
- [9] Buisson B., Gopalakrishnan M., Arneric S.P., Sullivan J.P., Bertrand D., Human $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptor in HEK 293 cells: A patch-clamp study, *J. Neurosci.* 16 (1996) 7880–7891.
- [10] Buisson B., Bertrand D., Open-channel blockers at the human $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptor, *Mol. Pharmacol.* 53 (1998) 555–563.
- [11] Buisson B., Gopalakrishnan M., Bertrand D., Stable expression of human neuronal nicotinic receptors in human embryonic kidney-293 cells, in: Arneric S.P., Brioni J.D. (Eds.), *Neuronal nicotinic receptors: pharmacology and therapeutic opportunities*, John Wiley & Sons Inc., New York, 1998, in press.
- [12] Burstein E.S., Spalding T.A., Brann M.R., Pharmacology of muscarinic receptor subtypes constitutively activated by G proteins, *Mol. Pharmacol.* 51 (1997) 312–319.
- [13] Cartaud A., Coutant S., Petrucci T.C., Cartaud J., Evidence for in situ and in vitro association between beta-dystroglycan and the subsynaptic 43K rapsyn protein. Consequence for acetylcholine receptor clustering at the synapse, *J. Biol. Chem.* 273 (1998) 11321–11326.
- [14] Changeux J.P., The acetylcholine receptor: A model for allosteric membrane proteins, *Biochem. Soc. Trans.* 23 (1995) 195–205.
- [15] Charpentier S., Jarvie K.R., Severynse D.M., Caron M.G., Tiberi M., Silencing of the constitutive activity of the dopamine D1 receptor. Reciprocal mutations between D1 receptor subtypes delineate residues underlying activation properties, *J. Biol. Chem.* 271 (1996) 28071–28076.
- [16] Clarke P.B.S., The fall and rise of neuronal alpha-bungarotoxin binding proteins, *Trends Pharmacol. Sci.* 13 (1992) 407–413.
- [17] Clarke P.B.S., Schwartz R.D., Paul S.M., Pert C.B., Pert A., Nicotinic binding in rat brain: autoradiographic comparison of [3 H]acetylcholine, [3 H]nicotine, and [125 I]-alpha-bungarotoxin, *J. Neurosci.* 5 (1985) 1307–1315.
- [18] Coggan J.S., Paysan J., Conroy W.G., Berg D.K., Direct recording of nicotinic responses in presynaptic nerve terminals, *J. Neurosci.* 17 (1997) 5798–5806.
- [19] Colledge M., Froehner S.C., Tyrosine phosphorylation of nicotinic acetylcholine receptor mediates Grb2 binding, *J. Neurosci.* 17 (1997) 5038–5045.
- [20] Colquhoun D., Sakmann B., Fluctuations in the microsecond time range of the current through single acetylcholine receptor ion channels, *Nature* 294 (1981) 464–466.
- [21] Corringer P.J., Galzi J.L., Eisele J.L., Bertrand S., Changeux J.P., Bertrand D., Identification of a new component of the agonist binding site of the nicotinic alpha 7 homooligomeric receptor, *J. Biol. Chem.* 270 (1995) 11749–11752.
- [22] Corriveau R.A., Berg D.K., Coexpression of multiple acetylcholine receptor genes in neurons: Quantification of transcripts during development, *J. Neurosci.* 13 (1993) 2662–2671.
- [23] Court J., Clementi F., Distribution of nicotinic subtypes in human brain, *Alzheimer Dis. Assoc. Disord.* 9 (1995) 6–14.
- [24] Couturier S., Bertrand D., Matter J.M., Hernandez M.C., Bertrand S., Millar N., Valera S., Barkas T., Ballivet M., A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX, *Neuron* 5 (1990) 847–856.
- [25] Czajkowski C., Karlin A., Structure of the nicotinic receptor acetylcholine-binding site. Identification of acidic residues in the delta subunit within 0.9 nm of the 5 alpha subunit-binding, *J. Biol. Chem.* 270 (1995) 3160–3164.
- [26] Daniels D.L., Cohen A.R., Anderson J.M., Brunger A.T., Crystal structure of the hCASK PDZ domain reveals the structural basis of class II PDZ domain target recognition, *Nat. Struct. Biol.* 5 (1998) 317–325.
- [27] Devillers-Thiéry A., Galzi J.L., Eiselé J.L., Bertrand S., Bertrand D., Changeux J.P., Functional architecture of the nicotinic acetylcholine receptor: a prototype of ligand-gated ion channels, *J. Membrane Biol.* 136 (1993) 97–112.
- [28] Edelstein S.J., Changeux J.P., Allosteric proteins after thirty years: The binding and state functions of the neuronal alpha 7 nicotinic acetylcholine receptors, *Experientia* 52 (1996) 1083–1090.
- [29] Eggerickx D., Denef J.F., Labbe O., Hayashi Y., Refetoff S., Vassart G., Parmentier M., Libert F., Molecular cloning of an orphan G-protein-coupled receptor that constitutively activates adenylate cyclase, *Biochem. J.* 309 (1995) 837–843.
- [30] Eilers H., Schaeffer E., Bickler P.E., Forsayeth J.R., Functional deactivation of the major neuronal nicotinic receptor caused by nicotine and a protein kinase C-dependent mechanism, *Mol. Pharmacol.* 52 (1997) 1105–1112.
- [31] Eiselé J.L., Bertrand S., Galzi J.L., Devillers-Thiéry A., Changeux J.P., Bertrand D., Chimeric nicotinic-serotonergic receptor combines distinct ligand binding and channel specificities, *Nature* 366 (1993) 479–483.
- [32] Elgoyhen A.B., Johnson D.S., Boulter J., Vetter D.E., Heinemann S., Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells, *Cell* 79 (1994) 705–715.
- [33] Franke C., Hatt H., Dudel J., Liquid filament switch for ultra-fast exchanges of solutions at excised patches of synaptic membrane of crayfish muscle, *Neurosci. Lett.* 77 (1987) 199–204.
- [34] Fu W.M., Liu J.J., Regulation of acetylcholine release by presynaptic nicotinic receptors at developing neuromuscular synapses, *Mol. Pharmacol.* 51 (1997) 390–398.
- [35] Fuhrer C., Sugiyama J.E., Taylor R.G., Hall Z.W., Association of muscle-specific kinase MuSK with the acetylcholine receptor in mammalian muscle, *EMBO J.* 16 (1997) 4951–4960.
- [36] Galzi J.L., Bertrand D., Devillers T.A., Revah F., Bertrand S., Changeux J.P., Functional significance of aromatic amino acids from three peptide loops of the alpha 7 neuronal nicotinic receptor site investigated by site-directed mutagenesis, *FEBS Lett.* 294 (1991) 198–202.
- [37] Galzi J.L., Bertrand S., Corringer P.J., Changeux J.P., Bertrand D., Identification of calcium binding sites that regulate potentiation of a neuronal nicotinic acetylcholine receptor, *EMBO J.* 15 (1996) 5824–5832.

- [38] Galzi J.L., Changeux J.P., Neuronal nicotinic receptors: Molecular organization and regulations, *Neuropharmacology* 34 (1995) 563–582.
- [39] Galzi J.L., Devillers-Thiéry A., Hussy N., Bertrand S., Changeux J.P., Bertrand D., Mutations in the channel domain of a neuronal nicotinic receptor convert ion selectivity from cationic to anionic, *Nature* 359 (1992) 500–505.
- [40] Galzi J.L., Edelstein S.J., Changeux J.P., The multiple phenotypes of allosteric receptor mutants, *Proc. Natl. Acad. Sci. USA* 93 (1996) 1853–1858.
- [41] Galzi J.L., Revah F., Black D., Goeldner M., Hirth C., Changeux J.P., Identification of a novel amino acid α -Tyr 93 within the active site of the acetylcholine receptor by photoaffinity labeling: additional evidence for a three-loop model of the acetylcholine binding site, *J. Biol. Chem.* 265 (1990) 10430–10437.
- [42] Gether U., Ballesteros J.A., Seifert R., Sanders-Bush E., Weinstein H., Kobilka B.K., Structural instability of a constitutively active G protein-coupled receptor. Agonist-independent activation due to conformational flexibility, *J. Biol. Chem.* 272 (1997) 2587–2590.
- [43] Gopalakrishnan M., Buisson B., Touma E., Giordano T., Campbell J.E., Hu I.C., Donnelly-Roberts D., Arneric S.P., Bertrand D., Sullivan J.P., Stable expression and pharmacological properties of the human $\alpha 7$ nicotinic acetylcholine receptor, *Eur. J. Pharmacol. Mol. Pharmacol. Sec.* 290 (1995) 237–246.
- [44] Gopalakrishnan M., Molinari E.J., Sullivan J.P., Regulation of human $\alpha 4 \beta 2$ neuronal nicotinic acetylcholine receptors by cholinergic channel ligands and second messenger pathways, *Mol. Pharmacol.* 52 (1997) 524–534.
- [45] Gray R., Rajan A.S., Radcliffe K.A., Yakehiro M., Dani J.A., Hippocampal synaptic transmission enhanced by low concentrations of nicotine, *Nature* 383 (1996) 713–716.
- [46] Gurantz D., Harootyan A.T., Tsien R.Y., Dionne V.E., Margiotta J.F., VIP modulates neuronal nicotinic acetylcholine receptor function by a cyclic AMP-dependent mechanism, *J. Neurosci.* 14 (1994) 3540–3547.
- [47] Harrison N.L., Majewska M.D., Harrington J.W., Barker J.L., Structure-activity relationships for steroid interaction with the gamma-aminobutyric acid A receptor complex, *J. Pharmacol. Exp. Ther.* 241 (1987) 346–353.
- [48] Heidmann T., Changeux J.P., Characterization of the transient agonist-triggered state of the acetylcholine receptor rapidly labeled by the noncompetitive blocker [3 H]chlorpromazine: additional evidence for the open channel conformation, *Biochemistry* 25 (1986) 6109–6113.
- [49] Henderson V.W., The epidemiology of estrogen replacement therapy and Alzheimer's disease, *Neurology* 48 (suppl. 7) (1997) S27–S35.
- [50] Hodgkin A.L., Huxley A.F., A quantitative description of membrane current and its application to conduction and excitation in nerve, *J. Physiol. (Lond.)* 117 (1952) 500–544.
- [51] Horch H., Sargent P.B., Synaptic and extrasynaptic distribution of two distinct populations of nicotinic acetylcholine receptor clusters in the frog cardiac ganglion, *J. Neurocytol.* 25 (1996) 67–77.
- [52] Horch H.L., Sargent P.B., Perisynaptic surface distribution of multiple classes of nicotinic acetylcholine receptors on neurons in the chicken ciliary ganglion, *J. Neurosci.* 15 (1995) 7778–7795.
- [53] Hsu Y.N., Edwards S.C., Wecker L., Nicotine enhances the cyclic AMP-dependent protein kinase-mediated phosphorylation of $\alpha 4$ subunits of neuronal nicotinic receptors, *J. Neurochem.* 69 (1997) 2427–2431.
- [54] Jackson M.B., Spontaneous openings of the acetylcholine receptor channel, *Proc. Natl. Acad. Sci. USA* 81 (1984) 3901–3904.
- [55] Kao P.N., Karlin A., Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystinyl residues, *J. Biol. Chem.* 261 (1986) 8085–8088.
- [56] Kirsch J., Betz H., Glycine-receptor activation is required for receptor clustering in spinal neurons, *Nature* 392 (1998) 717–720.
- [57] Koshland D., Nemethy G., Filmer D., Comparison of experimental binding data and theoretical models in proteins containing subunits, *Biochemistry* 5 (1966) 365–385.
- [58] Krause R.M., Buisson B., Bertrand S., Corringer P.-J., Galzi J.L., Changeux J.P., Bertrand D., Ivermectin: a positive allosteric effector of the $\alpha 7$ neuronal nicotinic acetylcholine receptors, *Mol. Pharmacol.* 53 (1998) 283–294.
- [59] Kuntzweiler T.A., Anderson D.J., Campbell J.E., Manelli A., Buisson B., Bertrand D., Arneric S.P., Donnelly-Roberts D., Characterization of recombinant human $\alpha 2 \beta 2$ and $\alpha 3 \beta 2$ neuronal nicotinic acetylcholine receptors, *Soc. Neurosci. Abstr.* (1997) 155.1.
- [60] Labarca C., Nowak M.W., Zhang H.Y., Tang L.X., Deshpande P., Lester H.A., Channel gating governed symmetrically by conserved leucine residues in the M2 domain of nicotinic receptors, *Nature* 376 (1995) 514–516.
- [61] Lambert J.J., Belelli D., Hill-Venning C., Callachan H., Peters J.A., Neurosteroid modulation of native and recombinant GABA_A receptors, *Cell Mol. Neurobiol.* 16 (1996) 155–174.
- [62] Le-Novère N., Changeux J.P., Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells, *J. Mol. Evol.* 40 (1995) 155–172.
- [63] Lefstin J.A., Yamamoto K.R., Allosteric effects of DNA on transcriptional regulators, *Nature* 392 (1998) 885–888.
- [64] Léna C., Changeux J., Mulle C., Evidence for 'preterminal' nicotinic receptors on GABAergic axons in the rat interpeduncular nucleus, *J. Neurosci.* 13 (1993) 2680–2688.
- [65] Léna C., Changeux J.P., Allosteric modulations of the nicotinic acetylcholine receptor, *Trends Neurosci.* 16 (1993) 181–186.
- [66] Léna C., Changeux J.P., Role of Ca^{2+} ions in nicotinic facilitation of GABA release in mouse thalamus, *J. Neurosci.* 17 (1997) 576–585.
- [67] Lindstrom J., Nicotinic acetylcholine receptors in health and disease, *Mol. Neurobiol.* 15 (1997) 193–222.
- [68] Majewska M.D., Harrison N.L., Schwartz R.D., Barker J.L., Paul S.M., Steroid hormone metabolites are barbiturate-like modulators of GABA receptor, *Science* 232 (1986) 1004–1007.
- [69] Margiotta J.F., Pardi D., Pituitary adenylate cyclase-activating polypeptide type I receptors mediate cyclic AMP-dependent enhancement of neuronal acetylcholine sensitivity, *Mol. Pharmacol.* 48 (1995) 63–71.
- [70] Marshall D.L., Redfern P.H., Wonnacott S., Presynaptic nicotinic modulation of dopamine release in the three ascending pathways studied by in vivo microdialysis: Comparison of naive and chronic nicotine-treated rats, *J. Neurochem.* 68 (1997) 1511–1519.
- [71] Mayer M.L., Benveniste M., Patneau D.K., Wyklicky L., Jr., Pharmacologic properties of NMDA receptors, *Ann. N.Y. Acad. Sci.* 648 (1992) 194–204.
- [72] McEwen B.S., Gonadal and adrenal steroids regulate neurochemical and structural plasticity of the hippocampus via cellular mechanisms involving NMDA receptors, *Cell Mol. Neurobiol.* 16 (1996) 103–116.

- [73] McGehee D.S., Heath M.J.S., Gelber S., Devay P., Role L., Nicotine enhancement of fast synaptic excitatory synaptic transmission in CNS by presynaptic receptors, *Science* 269 (1995) 1692-1696.
- [74] McGehee D.S., Role L.W., Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons, *Annu. Rev. Physiol.* 57 (1995) 521-546.
- [75] McMahon L.L., Yoon K.W., Chiappinelli V.A., Electrophysiological evidence for presynaptic nicotinic receptors in the avian ventral lateral geniculate nucleus, *J. Neurophysiol.* 71 (1994) 826-829.
- [76] Monod J., Wyman J., Changeux J.P., On the nature of allosteric transitions: a plausible model, *J. Mol. Biol.* 12 (1965) 88-118.
- [77] Moss S.J., McDonald B.J., Rudhard Y., Schoepfer R., Phosphorylation of the predicted major intracellular domains of the rat and chick neuronal nicotinic acetylcholine receptor alpha 7 subunit by cAMP-dependent protein kinase, *Neuropharmacology* 35 (1996) 1023-1028.
- [78] Mulle C., Benoit P., Pinset C., Roa M., Changeux J.P., Calcitonin gene-related peptide enhances the rate of desensitization of the nicotinic acetylcholine receptor in cultured mouse muscle cells, *Proc. Natl. Acad. Sci. USA* 85 (1988) 5728-5732.
- [79] Mulle C., Changeux J.P., A novel type of nicotinic receptor in the rat central nervous system characterized by patch-clamp techniques, *J. Neurosci.* 10 (1990) 169-175.
- [80] Mulle C., Léna C., Changeux J.P., Potentiation of nicotinic receptor response by external calcium in rat central neurons, *Neuron* 8 (1992) 937-945.
- [81] Numa S., Noda M., Takahashi H., Tanabe T., Toyosato M., Furutani Y., Kikuyotani S., Molecular structure of the nicotinic acetylcholine receptor, *Cold Spring Harb. Symp. Quant. Biol.* 48 (1983) 57-69.
- [82] Palma E., Bertrand S., Binzoni T., Bertrand D., Neuronal nicotinic alpha 7 receptor expressed in *Xenopus* oocytes presents five putative binding sites for methyllycaconitine, *J. Physiol. (Lond.)* 491.1 (1996) 151-161.
- [83] Pan Z.H., Zhang D., Zhang X., Lipton S.A., Agonist-induced closure of constitutively open gamma-aminobutyric acid channels with mutated M2 domains, *Proc. Natl. Acad. Sci. USA* 94 (1997) 6490-6495.
- [84] Peng X., Katz M., Gerzanich V., Anand R., Lindstrom J., Human alpha 7 acetylcholine receptor: cloning of the alpha 7 subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional alpha 7 homomers expressed in *Xenopus* oocytes, *Mol. Pharmacol.* 45 (1994) 546-554.
- [85] Pereira E.F., Alkondon M., Reinhardt S., Maelicke A., Peng X., Lindstrom J., Whiting P., Albuquerque E.X., Physostigmine and galanthamine: probes for a novel binding site on the alpha 4 beta 2 subtype of neuronal nicotinic acetylcholine receptors stably expressed in fibroblast cells, *J. Pharmacol. Exp. Ther.* 270 (1994) 768-778.
- [86] Prezeau L., Gomez J., Ahern S., Mary S., Galvez T., Bockaert J., Pin J.P., Changes in the carboxyl-terminal domain of metabotropic glutamate receptor 1 by alternative splicing generate receptors with differing agonist-independent activity, *Mol. Pharmacol.* 49 (1996) 422-429.
- [87] Puchacz E., Buisson B., Bertrand D., Lukas R.J., Functional expression of nicotinic acetylcholine receptors containing rat alpha 7 subunits in human SH-SY5Y neuroblastoma cells, *FEBS Lett.* 354 (1994) 155-159.
- [88] Pugh P.C., Corriveau R.A., Conroy W.G., Berg D.K., Novel subpopulation of neuronal acetylcholine receptors among those binding alpha-bungarotoxin, *Mol. Pharmacol.* 47 (1995) 717-725.
- [89] Puia G., Santi M.R., Vicini S., Pritchett D.B., Purdy R.H., Paul S.M., Seeburg P.H., Costa E., Neurosteroids act on recombinant human GABA_A receptors, *Neuron* 4 (1990) 759-765.
- [90] Ramarao M.K., Cohen J.B., Mechanism of nicotinic acetylcholine receptor cluster formation by rapsyn, *Proc. Natl. Acad. Sci. USA* 95 (1998) 4007-4012.
- [91] Revah F., Bertrand D., Galzi J.L., Devillers-Thiéry A., Mulle C., Hussy N., Bertrand S., Ballivet M., Changeux J.P., Mutations in the channel domain alter desensitization of a neuronal nicotinic receptor, *Nature* 353 (1991) 846-849.
- [92] Rodbell M., The complex regulation of receptor-coupled G-proteins, *Adv. Enzyme Regul.* 37 (1997) 427-435.
- [93] Role L.W., Berg D.K., Nicotinic receptors in the development and modulation of CNS synapses, *Neuron* 16 (1996) 1077-1085.
- [94] Rosenmund C., Westbrook G.L., Calcium-induced actin depolymerization reduces NMDA channel activity, *Neuron* 10 (1993) 805-814.
- [95] Rubin M.M., Changeux J.P., On the nature of allosteric transitions; implications of non exclusive ligand binding, *J. Mol. Biol.* 21 (1966) 265-274.
- [96] Ruiz M.L., Karpen J.W., Single cyclic nucleotide-gated channels locked in different ligand-bound states, *Nature* 389 (1997) 389-392.
- [97] Rupprecht R., The neuropsychopharmacological potential of neuroactive steroids, *J. Psychiatr. Res.* 31 (1997) 297-314.
- [98] Scheer A., Cotecchia S., Constitutively active G protein-coupled receptors: potential mechanisms of receptor activation, *J. Recept. Signal Transduct. Res.* 17 (1997) 57-73.
- [99] Schwartz R.D., Lehmann J., Kellar K.J., Presynaptic nicotinic cholinergic receptors labeled by ³H acetylcholine on catecholamine and serotonin axons in brain, *J. Neurosci.* 42 (1984) 1495-1498.
- [100] Séguéla P., Wadiche J., Dineley M.K., Dani J.A., Patrick J.W., Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium, *J. Neurosci.* 13 (1993) 596-604.
- [101] Sigel E., Buhr A., The benzodiazepine binding site of GABA_A receptors, *Trends Pharmacol. Sci.* 18 (1997) 425-429.
- [102] Smith S.S., Gong Q.H., Hsu F.-C., Markowitz R.S., Ffrench-Mullen J.M.H., Li X., GABA_A receptor $\alpha 4$ subunit suppression prevents withdrawal properties of an endogenous steroid, *Nature* 392 (1998) 926-930.
- [103] Stauffer D.A., Karlin A., Electrostatic potential of the acetylcholine binding sites in the nicotinic receptor probed by reactions of binding-site cysteines with charged methanethiosulfonates, *Biochemistry* 33 (1994) 6840-6849.
- [104] Steinlein O.K., Mulley J.C., Propping P., Wallace R.H., Phillips H.A., Sutherland G.R., Scheffer I.E., Berkovic S.F., A missense mutation in the neuronal nicotinic acetylcholine receptor alpha 4 subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy, *Nature Genet.* 11 (1995) 201-203.
- [105] Steinlein O.K., Magnusson A., Stoodt J., Bertrand S., Weiland S., Berkovic S.F., Nakken K.O., Propping P., Bertrand D., An insertion mutation of the CHRNA4 gene in a family with autosomal dominant nocturnal frontal lobe epilepsy, *Hum. Mol. Genet.* 6 (1997) 943-947.
- [106] Tierney M.L., Birnir B., Pillai N.P., Clements J.D., Howitt S.M., Cox G.B., Gage P.W., Effects of mutating leucine to threonine in the M2 segment of alpha1 and beta1 subunits

- of GABA_A $\alpha 1\beta 1$ receptors, *J. Membr. Biol.* 154 (1996) 11–21.
- [107] Tsigelny I., Sugiyama N., Sine S.M., Taylor P., A model of the nicotinic receptor extracellular domain based on sequence identity and residue location, *Biophys. J.* 73 (1997) 52–66.
- [108] Tytgat J., Hess P., Evidence for cooperative interactions in potassium channel gating, *Nature* 359 (1992) 420–423.
- [109] Ullian E.M., McIntosh J.M., Sargent P.B., Rapid synaptic transmission in the avian ciliary ganglion is mediated by two distinct classes of nicotinic receptors, *J. Neurosci.* 17 (1997) 7210–7219.
- [110] Unwin N., The nicotinic acetylcholine receptor at 9 Å resolution, *J. Mol. Biol.* 229 (1993) 1101–1124.
- [111] Unwin N., Acetylcholine receptor channel imaged in the open state, *Nature* 373 (1995) 37–43.
- [112] Valera S., Ballivet M., Bertrand D., Progesterone modulates a neuronal nicotinic acetylcholine receptor, *Proc. Natl. Acad. Sci. USA* 89 (1992) 9949–9953.
- [113] Vernallis A.B., Conroy W.G., Berg D.K., Neurons assemble acetylcholine receptors with as many as three kinds of subunits and can segregate subunits among receptor subtypes, *Neuron* 10 (1993) 451–464.
- [114] Vernino S., Amador M., Luetje C.W., Patrick J., Dani J.A., Calcium modulation and high calcium permeability of neuronal nicotinic acetylcholine receptors, *Neuron* 8 (1992) 127–134.
- [115] Wafford K.A., Thompson S.A., Thomas D., Sikela J., Wilcox A.S., Whiting P.J., Functional characterization of human gamma-aminobutyric acid A receptors containing the $\alpha 4$ subunit, *Mol. Pharmacol.* 50 (1996) 670–678.
- [116] Wess J., G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition, *FASEB J.* 11 (1997) 346–354.
- [117] Wevers A., Jeske A., Lobron C., Birtsch C., Heinemann S., Maelicke A., Schroder R., Schroder H., Cellular distribution of nicotinic acetylcholine receptor subunit mRNAs in the human cerebral cortex as revealed by non-isotopic in situ hybridization, *Brain. Res. Mol.* 25 (1994) 122–128.
- [118] Wonnacott S., Presynaptic nicotinic ACh receptors, *Trends Neurosci.* 20 (1997) 92–98.
- [119] Yakel J.L., Lagrutta A., Adelman J.P., North R.A., Single amino acid substitution affect desensitization of the 5-hydroxytryptamine type 3 receptor expressed in *Xenopus* oocytes, *Proc. Natl. Acad. Sci. USA* 90 (1993) 5030–5033.
- [120] Yang X.H., Criswell H.E., Breese G.R., Nicotine-induced inhibition in medial septum involves activation of presynaptic nicotinic cholinergic receptors on gamma-aminobutyric acid-containing neurons, *J. Pharmacol. Exp. Ther.* 276 (1996) 482–489.
- [121] Yoshihara C.M., Hall Z.W., Increased expression of the 43-kD protein disrupts acetylcholine receptor clustering in myotubes, *J. Cell. Biol.* 122 (1993) 169–79.
- [122] Zhang Z.W., Coggan J.S., Berg D.K., Synaptic currents generated by neuronal acetylcholine receptors sensitive to α -bungarotoxin, *Neuron* 17 (1996) 1231–1240.

Molecular dissection of subunit interfaces in the nicotinic acetylcholine receptor

Steven M. Sine, Nina Bren, Polly A. Quiram

Receptor Biology Laboratory, Department of Physiology and Biophysics, Mayo Foundation, Rochester, Minnesota 55905, USA

Abstract — Ligand binding sites in the muscle nicotinic acetylcholine receptor are generated by pairs of α and non- α subunits. The non- α subunits, γ , δ and ϵ , contribute significantly to overall affinity of agonists and antagonists, and confer selectivity of these ligands for the two binding sites. By constructing chimeras composed of segments of the various non- α subunits and determining ligand selectivity, we have identified four loops, well separated in the linear sequence, that contribute to the non- α portion of the binding site. Studies of point mutations in these loops and labeling of engineered cysteines show that the peptide backbones of each non- α subunit fold into similar basic scaffolds. Studies of mutations of the peptide antagonists α -conotoxin M1 and ImI reveal pairs of residues in the binding site and the toxin that stabilize the complex. (©Elsevier, Paris)

Résumé — Dissection moléculaire des interfaces entre sous-unités dans le récepteur nicotinique à l'ACh. Les sites de liaison des ligands cholinergiques au récepteur nicotinique musculaire résultent d'interactions entre des paires de sous-unités α et non- α . Les sous-unités γ , δ et ϵ contribuent ainsi à former deux sites de liaison d'affinité différente pour les agonistes et les antagonistes. En étudiant la sélectivité de liaison de chimères composées de segments des différentes sous-unités non- α , nous avons mis en évidence quatre boucles espacées dans la séquence primaire, qui participent à la partie non- α des sites de liaison. Des mutations ponctuelles dans ces boucles et le marquage de cystéines introduites par mutagenèse montrent que les peptides non- α se replient tous suivant le même motif. De plus, l'étude de mutations d'antagonistes peptidiques, les α -conotoxines M1 et ImI, montre que des interactions entre paires de résidus de la toxine et du site de liaison stabilisent le complexe. (©Elsevier, Paris)

molecular dissection / subunit interfaces / α -conotoxins / curare

1. Introduction

Acetylcholine receptors (AChR) from vertebrate skeletal muscle are pentamers of homologous subunits with compositions $\alpha_2\beta\gamma\delta$ in fetal or $\alpha_2\beta\epsilon\delta$ in adult muscle. The ligand binding sites are generated by apposition of pairs of non-equivalent subunits, $\alpha\delta$ and either $\alpha\gamma$ or $\alpha\epsilon$. Antagonists of the curare or α -conotoxin families select between the two sites of each receptor type, with the degree of selectivity ranging from 100- to 10 000-fold. Because the α subunit is common to each binding site, differences in affinity are attributed to different contributions of the non- α subunits. Over the past several years, we have used subunit chimeras and point mutations to determine the origin of the site-selectivities of these antagonists [2, 4, 5].

The present work describes identification of residues in the non- α subunits that confer site selectivity of curare for the adult muscle AChR. Cysteine mutagenesis is then used to identify residues in close proximity to the site of α -bungarotoxin binding. Finally, mutant α -conotoxins are used in combination with receptor mutations to probe binding sites in both the muscle and neuronal α_7 receptors. The results reveal pairs of residues in both the toxins and the receptors that interact to stabilize the α -conotoxin-receptor complex.

2. Materials and methods

2.1. Materials

Dimethyl-*d*-tubocurarine (DMT) was generously provided by the Eli Lilly Co. 125 I-labeled α -bungarotoxin (btx) was purchased from Dupont NEN, and the 293 human embryonic kidney cell line (293 HEK) from the American Type Culture Collection.

2.2. Plasmids and mutagenesis

Sources of the mouse AChR cDNAs and subcloning into the cytomegalovirus-based expression vector, pRBG4, were as described [4]. Human α_7 and rat 5HT-3 subunit cDNAs were generously provided by Drs. John Lindstrom and William Green. Chimeric subunit cDNAs were constructed by bridging naturally occurring or mutagenically installed restriction sites with synthetic double stranded oligonucleotides [4]. The chimeras are designated as follows: the first letter gives the subunit from which N-terminal sequence is taken, the following number gives the position of the chimeric junction, and the final letter gives the subunit from which C-terminal sequence is taken.

2.3. Expression of mutant receptors and ligand binding measurements

293 HEK cells were transfected with mutant or wild type AChR subunit cDNAs using calcium phosphate precipitation.

Three days after transfection, ligand binding to intact cells was measured by competition against the initial rate of ^{125}I - α -btx binding [4]. After harvesting, the cells were briefly centrifuged, resuspended in high potassium Ringer's solution, and divided into aliquots for ligand binding measurements. Specified concentrations of ligand were added 30 min prior to addition of ^{125}I - α -btx, which was allowed to bind for 30 min to occupy approximately half of the surface receptors. Binding was terminated by addition of 2 mL of potassium Ringer's solution containing 300 μM *d*-tubocurarine chloride. Cells were then harvested by filtration through Whatman GF-B filters using a Brandel Cell Harvester and washed four times with 3 mL of potassium Ringer's solution. Non-specific binding was determined in the presence of 10 mM carbamylcholine. The initial rate of α -btx binding was calculated to yield fractional occupancy by competing ligand. Binding measurements were analyzed by fitting to either the monophasic Hill equation or the sum of two distinct binding sites using the program UltraFit (BIOSOFT).

2.4. Synthesis and purification of α -conotoxin ImI and derivatives

α -Conotoxins (CTx) were synthesized by standard FMOC (9-fluorenyl-methoxycarbonyl) chemistry on an Applied Biosystems 431A peptide synthesizer. During synthesis, cysteine (S-triphenylmethyl) protecting groups were incorporated at cysteines 3 and 12, and acetamidomethyl (ACM) protecting groups were incorporated at cysteines 2 and 8. The linear peptide was purified by reversed phase HPLC using a Vydac C18 preparative column with TFA/acetonitrile buffers. The two intramolecular disulfide bridges were formed as follows: the cysteine S-triphenylmethyl protecting groups of cysteines 3 and 12 were removed during TFA cleavage of the linear peptide from the support resin, and the peptide was oxidized by molecular oxygen to form the 3–12 disulfide by stirring in 50 mM ammonium bicarbonate buffer, pH 8.5 at 25 °C for 24 h. The peptide was lyophilized prior to the formation of the second bridge. The ACM protecting groups on cysteine 2 and 8 were removed oxidatively by reaction with iodine for 16 h prior to carbon tetrachloride extraction. Residual iodine was separated from the pure product by HPLC. Formation of disulfide bonds was confirmed by lack of reaction with Ellman's reagent, and molecular mass was confirmed by mass spectrometry.

3. Results

3.1. Identification of residues in the adult muscle AChR that confer selectivity for DMT-

Previous work showed that the pair of equivalent residues $\gamma\text{Y117}/\delta\text{T119}$ are major determinants of DMT selectivity in the fetal AChR [4]. γY117 contributes to high affinity of the $\alpha\gamma$ site, whereas δT119 contributes to low affinity of the $\alpha\delta$ site. Because serine occupies the equivalent position in the ϵ subunit of the adult AChR, and high affinity requires an aromatic side chain at this position, the $\alpha\epsilon$ site

is not expected to bind DMT with high affinity. To determine the origin of high affinity conferred by the ϵ subunit, we constructed a series of ϵ - δ chimeras (figure 1), coexpressed them with complementary subunits and measured DMT binding by competition against the initial rate of ^{125}I - α -btx binding [1].

Selectivity of the adult AChR for DMT is illustrated in figure 1; distinct affinities of the $\alpha\epsilon$ and $\alpha\delta$ sites are clearly resolved, with the two-site fit disclosing affinities different by 70-fold. Substituting ϵ sequence into the N-terminal 63 positions of the δ subunit increases DMT affinity to approach that conferred by the pure ϵ subunit (figure 1, $\epsilon 63\delta$). Conversely, substituting δ sequence between positions 43 and 63 of the ϵ subunit decreases affinity to approach that of the pure δ subunit (figure 1, $\epsilon 43\delta 63\epsilon$). Thus, determinants of DMT selectivity in the adult AChR are located in the major extracellular domain between residues 43 and 63 of the ϵ subunit and equivalent residues of the δ subunit.

To identify the key residues within the 43 to 63 segment, we constructed additional ϵ - δ chimeras and point mutants in the ϵ and δ subunits. The overall results show that two pairs of equivalent residues, $\epsilon\text{158}/\delta\text{H60}$ and $\epsilon\text{D59}/\text{A61}$, fully account for site-selectivity of DMT for the adult receptor. To confirm that these pairs of residues are solely responsible for DMT selectivity of the adult receptor, we expressed the double mutant subunits, ϵ (I58H + D59A) and δ (H60I + A61D), alone or together, and measured DMT binding. Incorporating both the ϵ and δ double mutants into a single receptor mimics the selective binding of DMT to the wild type adult receptor [1]. Thus, the pair of equivalent residues $\epsilon\text{158}/\delta\text{H60}$ and $\epsilon\text{D59}/\text{A61}$ account entirely for DMT selectivity of the adult receptor.

3.2. Probing the non- α subunits for residues close to the site of α -btx binding

To identify residues in the non- α subunits close to the site of α -btx binding, we carried out cysteine mutagenesis of the predisulfide region of the γ subunit (residues 116–121). As this region contains endogenous cysteines at positions 106 and 115, these were first mutated to serine, and the remaining residues individually mutated to cysteine. We applied one of three methanethiosulfonate reagents to HEK cells expressing each mutant subunit plus complementary α , β and δ subunits, and measured the total number of ^{125}I - α -btx sites. Of the 18 combinations of mutant and reagent, only receptors harboring γL119C treated with the quaternary ammonium reagent MTSET show a significant decrease of the total number of α -btx sites (table I). Neither the positively charged aminoethyl (MTSEA) nor the nega-

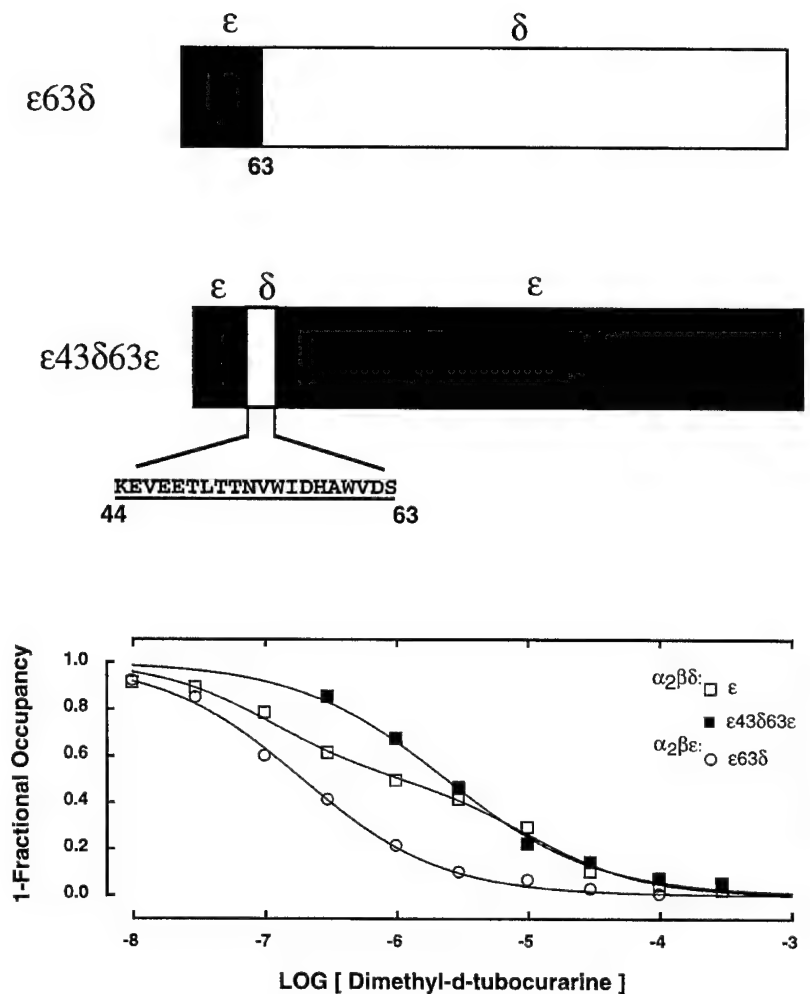


Figure 1. DMT binding to receptors containing ϵ - δ chimeras. Upper panels are schematic drawings of the ϵ - δ subunit chimeras: $\epsilon 63\delta$ contains ϵ sequence from the N-terminus to position 63 followed by δ sequence to the C-terminus, whereas $\epsilon 43\delta 63\epsilon$ contains δ sequence between residues 43 and 63 of the ϵ subunit. Shaded portions represent ϵ sequence, and unshaded portions δ sequence. Bold underlined text indicates δ sequence. Lower panel: binding of DMT to surface receptors with the indicated compositions: wild type ϵ (open squares), $\epsilon 43\delta 63\epsilon$ (filled squares), and $\epsilon 63\delta$ (open circles). For wild type, the curve through the data is a least squares fit to a two-site equation with the fitted parameters $K_A = 0.11 \mu\text{M}$ and $K_B = 10.5 \mu\text{M}$, and the fraction of each site constrained to 0.5. For receptors containing chimeric subunits, the curves through the data are fits to the Hill equation with the following fitted parameters: $\epsilon 43\delta 63\epsilon$, $K_{\text{App}} = 2.4 \mu\text{M}$ and $n = 0.76$; $\epsilon 63\delta$, $K_{\text{App}} = 0.28 \mu\text{M}$ and $n = 0.90$.

tively charged sulfonatoethyl (MTSES) reagents affect the number of α -btx sites. The decrease of approximately 50% of α -btx sites suggests that MTSET selectively blocks one of the two ligand binding sites in γ L119C receptors.

3.3. MTSET selectively modifies cysteines at equivalent positions of the ϵ and δ subunits

The high degree of homology among AChR subunits suggests that the polypeptide chains of each subunit fold into similar basic scaffolds. Support for this basic scaffold hypothesis comes from the observation that site-selectivity of DMT, CTx and carbamylcholine can be exchanged between the γ and δ subunits by exchanging a small number of residues at equivalent positions of the primary sequence. To determine whether residues in equivalent positions of the δ and ϵ subunits are functionally equivalent to

γ L119C, receptors containing either ϵ L119C or δ L121C were treated with MTSET and the total number of α -btx sites was measured. As observed for receptors containing γ L119C, MTSET reduces by 50% the total number of α -btx sites in receptors containing either δ L121C or ϵ L119C, but has no effect on receptors containing β Q119C or α T119C (table I).

3.4. Probing the neuronal α_7 binding site with CTx ImI and synthetic mutants

α -Conotoxins offer the opportunity to modify both the competing ligand and the receptor binding site to identify pairs of residues that stabilize the toxin-receptor complex. To begin study of the α_7 neuronal receptor binding site, we introduced conservative substitutions for each non-cysteine residue in CTx ImI and measured binding of each mutant toxin to α_7 /5HT-3 receptors expressed in 293 HEK

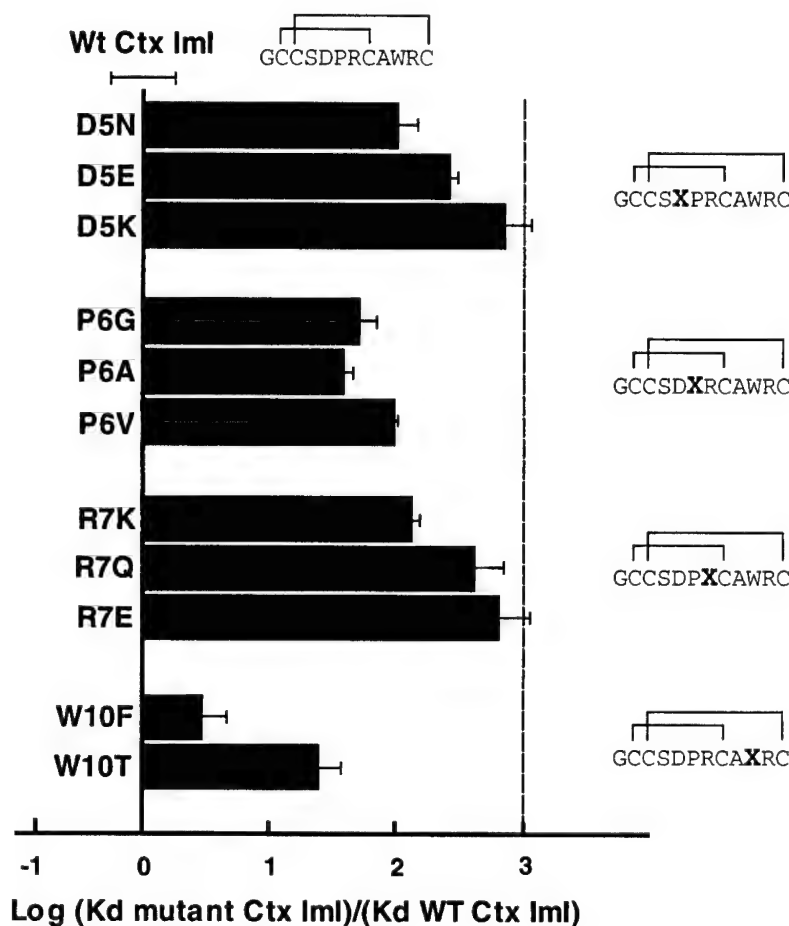


Figure 2. Side chain specificity of the determinants of CTx ImI affinity. For each CTx ImI mutant, dissociation constants are expressed as the log ratio relative to wild type CTx ImI. The affinity of wild type CTx ImI for $\alpha_7/5HT-3$ receptors is shown by the vertical bold line, and the error bars indicate \pm SD. In the schematic representations to the right, X indicates the mutant residue.

cells [3]. The results reveal two key regions in CTx ImI essential for high affinity binding (figure 2). The first region is the triad Asp-Pro-Arg in the first loop of CTx ImI, where individual mutations decrease affinity by 70- to 500-fold. The second region is the single tryptophan in the second loop, which when mutated to threonine decreases affinity by 30-fold. On the other hand, mutating the four remaining non-cysteines in CTx ImI does not significantly alter affinity for $\alpha_7/5HT-3$ receptors. These null mutations include acetylation of the amino-terminal glycine and neutralization of arginine in the second loop (R11Q). Thus mutation of four of the eight non-cysteines in CTx ImI alters affinity for $\alpha_7/5HT-3$ receptors.

We carried out systematic mutations of each of the four bioactive residues in CTx ImI. The overall results reveal that within the first loop, the triad Asp-Pro-Arg is conformationally restricted by the proline, and that the flanking charged residues require maintenance of both charge sign and side chain length (figure 2). Within the second loop, mutation of the

lone tryptophan shows that it contributes its aromatic ring to stabilize the α_7 -CTx ImI complex.

4. Discussion

The experiments described herein probe the structure of the ligand binding site of the nicotinic AChR using: 1) chimeric subunits combined with the site-selective competitive antagonist DMT; 2) cysteine mutagenesis followed by reaction with methylthio-sulfonate reagents and measurements of the number of α -btx binding sites; and 3) mutagenesis of α -CTx ImI followed by measurements of binding to $\alpha_7/5HT-3$ chimeric receptors.

Studies of chimeric ϵ - δ subunits reveal residues in the ϵ and δ subunits that give the two binding sites of the adult mouse AChR different affinities for the curariform antagonist DMT. Previous work identified a different set of residues in the γ and δ subunits that confer site-selectivity in the fetal mouse AChR [4]. Two sets of determinants were

Table I. Total α -bungarotoxin sites following methanethio-sulfonate treatment.

Mutant	α -btx binding sites (%)		
	MTSET	MTSEA	MTSES
<i>Fetal AChR:</i>			
$\alpha\beta\gamma\delta$	102 \pm 3	107 \pm 4	103 \pm 4
γ C106S + C115S	95 \pm 7	105 \pm 3	103 \pm 7
γ I116C	98 \pm 3	105 \pm 1	103 \pm 1
γ Y117C	86 \pm 11	94 \pm 1	105 \pm 6
γ W118C	85 \pm 1	105 \pm 2	112 \pm 7
γ L119C	43 \pm 6	94 \pm 10	103 \pm 2
γ P120C	100 \pm 5	99 \pm 2	105 \pm 1
γ P121C	97 \pm 5	101 \pm 6	96 \pm 1
<i>Adult AChR:</i>			
$\alpha\beta\epsilon\delta$	99 \pm 3	117 \pm 6	—
α T119C	97 \pm 6	98 \pm 3	—
β Q119C	98 \pm 8	100 \pm 13	—
ϵ L119C	50 \pm 4	94 \pm 5	—
δ L119C	52 \pm 4	96 \pm 11	—

The indicated mutant subunit cDNAs were coexpressed with complementary wild type subunit cDNAs. The total number of α -btx sites is expressed as a percentage of that determined for the corresponding untreated receptor. Values are means \pm standard deviations for 3–12 determinations.

identified, with each set flanking the disulfide loop common to all members of the AChR superfamily; the predisulfide set is γ I116/ δ V118 and γ Y117/ δ T119 and the postdisulfide set is γ S161/ δ K163. By contrast, selectivity determinants in the adult receptor are far from these in the linear sequence, and comprise ϵ I58/ δ H60 and ϵ D59/ δ A61, showing that alternative residues confer DMT selectivity in fetal and adult receptors. The results support a basic scaffold hypothesis because selectivity can be exchanged between the ϵ and δ subunits by exchanging a small number of residues at equivalent positions of the primary sequence.

The cysteine mutagenesis experiments identify a localized region of the γ , ϵ and δ subunits that contributes to the site of α -btx binding. This region is located within one of four loops of the γ , ϵ and δ subunits that contribute to binding of agonists and competitive antagonists. Cysteines placed at the equivalent positions γ L119, δ L121 and ϵ L119 are chemically modified in a site-specific manner by MTSET, and the modifications block binding of α -btx to the binding site harboring the mutation. Prior to modification, cysteine mutant subunits assemble normally with complementary wild type subunits, and form binding sites with high affinity for α -btx and normal selectivities for the competitive antagonists DMT and CTx. Following modification by MTSET, α -btx no longer

binds with high affinity to sites harboring γ L119C. The results support the hypothesis that residues at equivalent positions in the primary sequences occupy equivalent positions in three-dimensional space. In addition to supporting the basic scaffold hypothesis, the findings demonstrate that the γ , δ and ϵ subunits contribute to one face to the binding site, whereas equivalent portions of the α and β subunits do not. The overall findings indicate that γ L119 is close to or within the site of α -btx binding, and that MTSET modification of γ L119C creates a localized point of interaction with α -btx that prevents its binding.

Studies of mutant α -conotoxins reveal two regions of CTx ImI that confer specificity for α_7 /5HT-3 chimeric receptors. The first region is the conformationally sensitive triad Asp-Pro-Arg within the first loop of CTx ImI. Subtle changes in side chain length of the aspartic acid and arginine reduce affinity, and their side chains appear to be held in place by the intervening proline. Thus, the triad must maintain a specific conformation to fit properly into a specific and focal counterpart in the α_7 binding site. The second region is the tryptophan at position 10 within the second loop of CTx ImI. Studies of side chain specificity at position 10 indicate the requirement of an aromatic ring. Current mutant cycles analysis with mutant toxins and receptors aim to identify pairs of residues that stabilize the toxin-receptor complex.

References

- [1] Bren N., Sine S.M., Identification of residues in the adult nicotinic acetylcholine receptor that confer selectivity for curariform antagonists, *J. Biol. Chem.* 272 (1997) 30793–30798.
- [2] Prince R.J., Sine S.M., Molecular dissection of subunit interfaces in the acetylcholine receptor: Identification of residues that determine agonist selectivity, *J. Biol. Chem.* 271 (1996) 25770–25777.
- [3] Quiram P., Sine S.M., Structural elements in α -conotoxin ImI essential for binding to neuronal α_7 receptors, *J. Biol. Chem.* 273 (1998) 11007–11011.
- [4] Sine S.M., Molecular dissection of subunit interfaces in the acetylcholine receptor: Identification of residues that determine curare selectivity, *Proc. Natl. Acad. Sci. USA* 90 (1993) 9436–9440.
- [5] Sine S.M., Kreienkamp H.-J., Bren N., Maeda R., Taylor P., Molecular dissection of subunit interfaces in the acetylcholine receptor: Identification of determinants of α -Conotoxin M1 selectivity, *Neuron* 15 (1995) 205–211.
- [6] Sine S.M., Identification of equivalent residues in the γ , δ , and ϵ subunits of the nicotinic receptor that contribute to α -btx binding, *J. Biol. Chem.* 272 (1997) 23521–23527.

Functional determinants by which snake and cone snail toxins block the $\alpha 7$ neuronal nicotinic acetylcholine receptors

Denis Servent^a, Hung Lam Thanh^a, Stéphanie Antil^a, Daniel Bertrand^b,
Pierre-Jean Corringer^c, Jean-Pierre Changeux^b, André Ménez^a

^aDépartement d'Ingénierie et d'Etudes des Protéines, Bât 152, CEA-Saclay, 91191 Gif-sur-Yvette cedex, France

^bDépartement de Physiologie, CMU, 1211 Genève 4, Switzerland

^cLaboratoire de Neurobiologie Moléculaire, Institut Pasteur, 75724 Paris cedex 15, France

Abstract — Snakes and cone snails produce toxins which block muscular and/or neuronal nicotinic acetylcholine receptors (AChRs). This paper mostly focuses on the determinants by which a snake long chain curare-mimetic toxin and the cone snail toxin ImI bind specifically to the $\alpha 7$ neuronal receptor. In both cases, the site involves a small turn-like structure constrained by two half-cystines. (©Elsevier, Paris)

Résumé — Déterminants fonctionnels de toxines de serpents et de cônes qui bloquent le récepteur nicotinique de l'acétylcholine de type $\alpha 7$. Les serpents et les cônes marins produisent des toxines qui bloquent les récepteurs cholinergiques nicotiniques (AChRs) musculaires et/ou neuronaux. Nous avons identifié les déterminants majeurs par lesquels une toxine longue curarisante de serpents et la toxine de cônes ImI, reconnaissent le récepteur neuronal $\alpha 7$. Dans les deux cas, le site comporte une petite structure organisée en coude, encadrée par deux demi-cystines. (©Elsevier, Paris)

nicotinic acetylcholine receptors / snake curare-mimetic toxins / α -conotoxins

1. Introduction

Although they are phylogenetically distant, chordates (snakes) and molluscs (cone snails) include venomous species which elaborate various toxins that block muscular and/or neuronal nicotinic acetylcholine receptors (AChRs) [6, 15]. Understanding of the mode of action of these toxins, at the molecular level, requires elucidation of both their architecture and residues that are directly implicated in the specific recognition of AChRs. By introducing appropriate substitutions in amino acid sequences, we have elucidated: i) the specific functional elements which provide long chain curare-mimetic toxins from snakes with high affinity for $\alpha 7$ neuronal AChR; and ii) functional residues by which the conotoxin α -ImI from *Conus imperialis* recognizes $\alpha 7$ neuronal receptor. Evidence suggests that this specific recognition involves in both cases a small turn-like structure that may differ from the determinants by which snake and cone snail toxins bind to *Torpedo* AChRs.

2. Materials and methods

2.1. Solid phase peptide synthesis

Conotoxin α -ImI and its analogues were synthesized by solid phase using the Fmoc-chemistry. The SPPS was carried out on an ABI 430A automated synthesizer (Perkin-Elmer/Applied Biosystems Division) with the HBTU/HOBt *Fast-*

moc coupling program [7]. Pairing of the two disulphide bridges was achieved stepwise by specific chemistry as described previously [12]. The production yield of the α -ImI conotoxin and analogues was 10% as calculated from the starting Fmoc-Rink-AM- amide resin obtained from Novabiochem. Synthetic α -ImI and analogues were characterized by amino acid sequence analysis, analytical HPLC and electro spray ionization mass spectrometry (not shown).

2.2. $\alpha 7$ expression in HEK cells

The chimeric $\alpha 7$ -5HT3 receptor was expressed transiently in HEK cells after calcium precipitation as described previously [2].

2.3. α -Cobratoxin expression, purification and mutagenesis

The cDNA encoding α -CbtX was subcloned into pCP vector (fusion ZZ-CbtX) as previously described [3, 4], expressed into the BL21 *E. coli* strain and the recombinant fused toxin was purified onto a IgG-Sepharose column and treated in 0.1 N HCl with CNBr. The toxin was refolded in phosphate buffer (0.1 M, pH 7.8) containing GSH/GSSG (4 mM/2 mM) and purified to homogeneity on a reverse phase (C4) column. The α -CbtX mutant described here was prepared using the Stratagene kit (Quick Change) and the sequence of the entire gene was checked by automatic sequencing (ABI PRISM™ 310 Genetic Analyses, Applied Biosystem).

2.4. Binding assays

We determined the effect of increasing concentrations of: i) the wild-type and derivatized α -CbtX; and ii) α -ImI and its analogues, on the initial rate of [¹²⁵I]- α -Bgtx binding to the

$\alpha 7$ -5HT3 chimeric receptor [18]. The competitors were preincubated at different concentrations for at least 45 min with the cells suspension and filtrated 6 min after addition of 5 nM of ^{125}I - α -Bgtx. The protection constant calculated by fitting the competition data by the Hill equation reflects the K_d value [21].

3. Results and discussion

3.1. On the determinants by which long chain curare-mimetic toxins bind to $\alpha 7$ receptor with high affinity

Snake curare-mimetic toxins include both long chain toxins (66-74 residues and five disulphide bonds) and short chain toxins (60-62 residues and four disulphide bonds). They are all folded into three adjacent loops which emerge from a small globular core. The loops are rich in β -pleated sheet which provides the toxins with a leaf-like shape and two faces. While both short chain and long chain curare-mimetic toxins from snakes bind to *Torpedo* AChR with high affinity [6], only long chain toxins display high affinities for neuronal $\alpha 7$ receptor [18].

Two lines of evidence indicate that this property is associated with the presence of the extra disulfide bond (Cys26-Cys30), called the fifth disulphide, that is uniquely found in long chain toxins. First, selective reduction of the fifth bond of α -cobratoxin (α -Cbtx), a prototype of long chain toxins, followed by modification of its two free cysteines by a chemical reagent, caused a substantial affinity decrease for the $\alpha 7$ receptor without modifying the binding property of the toxin for the *Torpedo* AChR [18]. Second, mutation of each of the two extra half-cystines into serine of α -Cbtx caused a 25-fold affinity decrease uniquely for the $\alpha 7$ receptor (figure 1, table I). Therefore, we suggest that the fifth disulphide controls an appropriate conformation of some key residues, most probably belonging to the small loop 26-30, and which may interact with the $\alpha 7$ receptor. This loop adopts a β -turn structure [1], whose residues (Asp²⁷-Ala²⁸-Phe²⁹) protrude at the tip of the toxin central loop. Whether additional determinants contribute to the high affinity binding of long chain toxins to AChR remains to be determined.

The determinants by which long chain curare-mimetic toxins bind to muscular-type *Torpedo* AChR have not yet been elucidated. In contrast, the functional architecture of an erabutoxin from *Laticanda semifasciata* (Ea), short chain toxin is becoming well documented [5, 14, 20]. It involves at least 10 functionally important residues which constitute a homogeneous surface located only on the concave face of the toxin but spread on its three loops. Most of the functionally important residues of Ea that are located on the second and third loops of the toxin are highly conserved in most long chain toxins, suggesting that they are also part of the

site by which long chain curare-mimetic toxins bind to muscular-type *Torpedo* AChR. In agreement with this proposal are the numerous chemical modifications that were previously performed on various long chain curare-mimetic toxins [6]. Therefore, the small β -turn region which contributes to the high affinity binding of long-chain toxin to $\alpha 7$ receptor is adjacently located to the conserved functional determinants of curare-mimetic toxins.

3.2. On the determinants by which α -conotoxin ImI binds to $\alpha 7$ receptor

Cone snails are recognized for their remarkable capacity to elaborate a diversity of toxins with a large spectrum of biological activities [15]. Like snakes, they produce toxins that act on muscular and/or neuronal receptors AChRs. However, in sharp contrast to reptile toxins, cone snail toxins are usually small peptides which often contain less than 20 residues and two disulphide bonds which define two small loops [13]. The functional sites of conotoxins acting on muscular AChRs have been previously subjected to extensive studies [8, 19] whereas the functional anatomies of neuronal conotoxins remain unknown.

α -ImI is a 12 residue-containing peptide with two disulphide bonds, isolated from venom of *Conus imperialis* [13]. This peptide has been described as a specific ligand of some neuronal AChRs, including the $\alpha 7$ subtype [11]. To identify the site by which this peptide binds to this neuronal AChR, we substituted all its positions, but the four half-cystines, by an alanine residue and determined the affinity constants of the analogues for the $\alpha 7$ neuronal AChR, using as receptor the chimeric construction previously described [2]. The two disulphide bonds of α -ImI analogues were paired as in the native toxin. In addition, however, we prepared two peptides, called I and II which respectively possessed none and one disulphide bond (Cys²SSCys⁸).

The K_d value for the synthetic α -ImI was 4 μM (figure 2, table I), in agreement with previous result obtained for chick muscle $\alpha 7$ receptors expressed during development [17]. This value, however, was approximately 20-40-fold higher as compared to that determined from electrophysiological recordings for mammalian (rat or mouse) $\alpha 7$ receptors [11, 16]. The competition curves performed with the alanine-containing analogues are shown in figure 2 and the derived inhibition constants are reported in table I. The substitutions G1A, S4A and R11A virtually unaffected the competition potency of α -ImI. In contrast, the mutation W10A caused a six-fold affinity decrease and the substitutions D5A, P6A and R7A, caused respectively 38-fold, 70-fold and 165-fold affinity decreases.

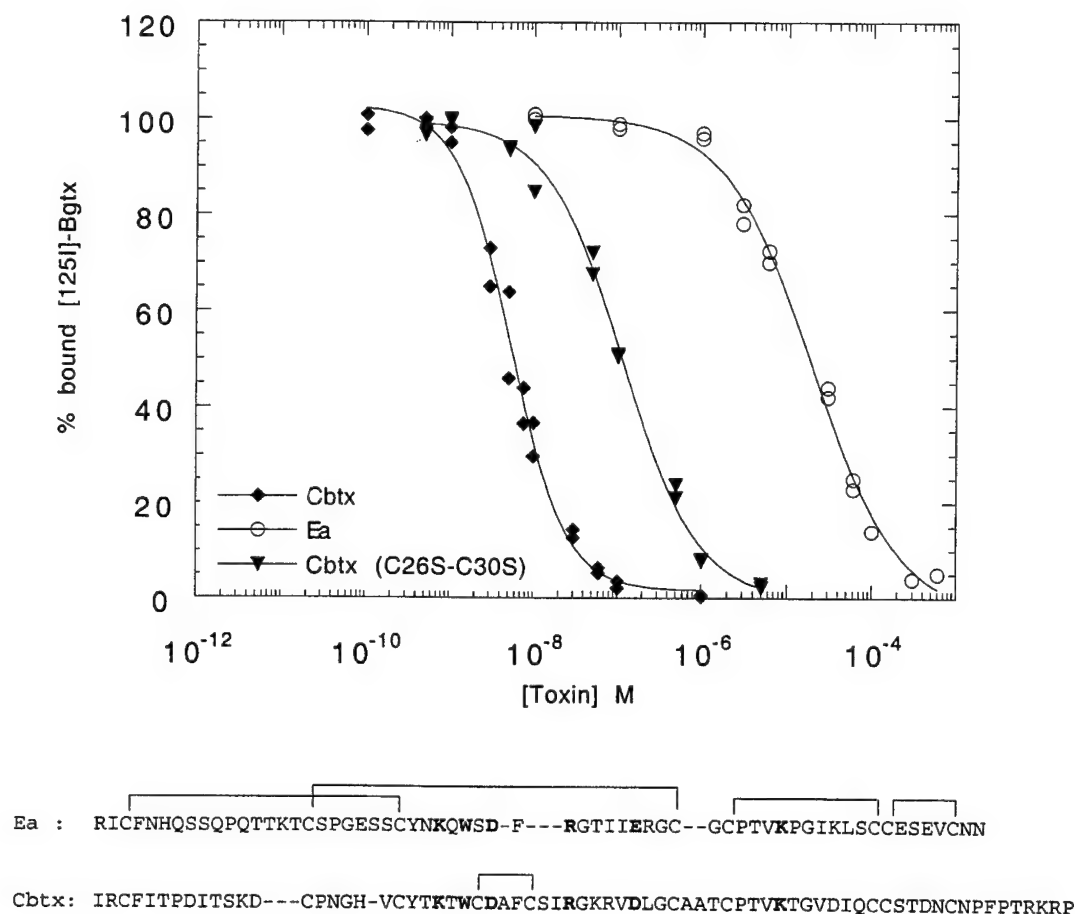


Figure 1. Inhibition of binding of ^{125}I - α -bungarotoxin to the chimeric $\alpha 7$ receptor ($\alpha 7$ -V201-5HT $_3$) [2], by: i) wild type α -cobratoxin (Cbtx); ii) Cbtx whose both half cystines C26 and C30 were replaced by a serine; and iii) erabutoxin a (Ea). The recombinant receptor was produced at the surface of HEK 293 cells. Varying concentrations of the competitors were incubated for 45 min with cell suspension and the mixtures were filtered through GF/C filters, 6 min after addition of ^{125}I - α -Bgtx at a final concentration of 5 nM. The continuous lines correspond to theoretical dose-response curves fitted through the data points using the non-linear Hill equation. The amino acid sequences of a short chain toxin (Ea) and a long chain toxin (Cbtx) are presented under the figure. For sake of clarity, the four disulphide bonds that are conserved in all curaremimetic toxins have been presented in Ea sequence only. The fifth disulphide that is only found on long chain toxins is also shown in the Cbtx sequence. Bold letters indicate the functional residues of Ea that are conserved in at least 90% of 84 amino acid sequences of short chain and long chain curaremimetic toxins [6]. These functionally highly conserved residues are Lys-27, Trp-29, Asp-31, Arg-33, Glu (or Asp)-38 and Lys-47 (using the numbering in the amino acid sequence of Ea).

The sequence D5-P6-R7 therefore, is a major determinant which, assisted by W10, allows α -ImI to bind to the $\alpha 7$ receptor.

Two lines of evidence further support the view that the sequence D5-P6-R7 is functionally important. First, introduction of the additional individual substitutions R7K, R7Nle, D5N and P6S also caused affinity decreases (see *table I*). Second, suppression of the bond Cys3-Cys12 had no effect on the toxin affinity whereas the additional deletion of the bond

Cys2-Cys8, which is suspected to control the local organization of the sequence D5-P6-R7 (see below), substantially affected toxin affinity (*table I*).

What is the structural organization associated with the functionally critical sequence D5-P6-R7? At present, the three-dimensional structure of α -ImI is unknown. However, as inferred from an unpublished modeling study, based on both the known architectures of conotoxins sharing the same disulphide framework, such as α -conotoxin G1 [9] and secondary structure prediction

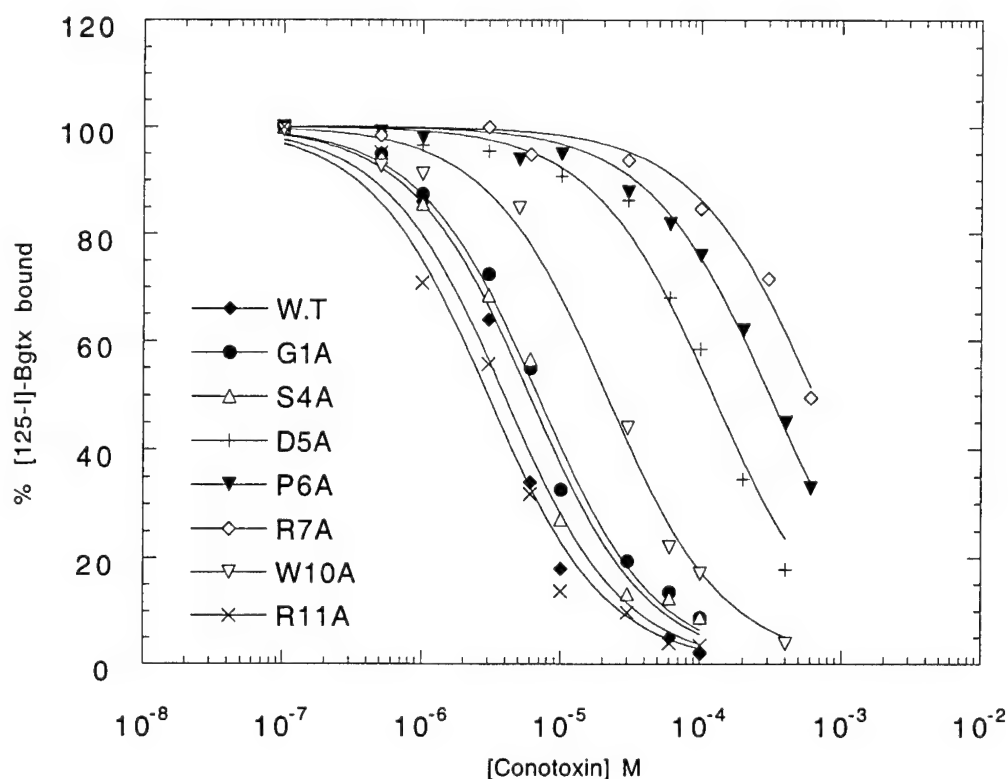


Figure 2. Inhibition of binding of ^{125}I - α -bungarotoxin to the chimeric $\alpha 7$ receptor by the wild type α -ImI conotoxin and its alanine-containing analogues. Binding experiments were performed as described in the legend to figure 1. The amino acids sequence and disulfide bond pairing of α -conotoxins ImI are presented.

methods [10], the region 5-8 is anticipated to adopt a β turn-like structure.

Previous studies have shown that the functional site by which α -G1 recognizes the muscular-type AChR involves primarily residues that are located between the half-cystines 8 and 13 [8, 19]. In contrast, we have shown that the $\alpha 7$ neuronal AChR-specific binding site of ImI is predominantly located in the small loop cyclized by the two half-cystines 2 and 8. Therefore, the major determinants by which α -conotoxins preferentially bind to $\alpha 7$ neuronal and muscular-type AChRs seem to be differently located on the conotoxin scaffold.

4. Concluding remarks

At least two types of toxins have some selectivity toward the $\alpha 7$ receptor. These are the snake long

chain toxins, including curare-mimetic toxins and K-neurotoxins [18] and some cone snail toxins, like ImI [11]. Though the two types of toxins share no three-dimensional analogy, their capacity to recognize the neuronal $\alpha 7$ receptor seems to be primarily associated with presentation of few specific critical residues on a small β -turn structure, stabilized by two adjacent half-cystines. Whether or not these structurally similar functional determinants recognize the same region of the $\alpha 7$ receptor remains to be determined.

Acknowledgments

This work was supported by a grant from AFM, Paris. We are deeply indebted to Dr D. Gordon for reading and criticizing the manuscript.

Table I. Affinity constants of α -Cbtx, α -ImI and their substituted analogues for the $\alpha 7$ receptor. Cbtx and Cbtx (C26S-C30S), stand respectively for α -cobratoxin and its mutant in which each of its two disulphide bonds 26 and 30 were mutated into serine. The peptides I and II correspond respectively to the α -conotoxin ImI in which: i) the two disulphide bonds were reduced and acetamidomethylated; and ii) only the disulphide 3-12 was reduced and subsequently modified.

	Kp (μM)	Kp mutant/ Kp W.T
<i>Cbtx</i>		
Wild-type	0.0055 ± 0.001	1
Cbtx (C26S-C30S)	0.125 ± 0.015	23
<i>α-ImI</i>		
Wild-type	4 ± 0.26	1
G1A	5.6 ± 0.6	1.4
S4A	5.3 ± 0.8	1.3
D5A	150 ± 28	37.5
P6A	280 ± 151	70
R7A	660 ± 235	165
W10A	24 ± 3.1	6
R11A	2.9 ± 0.6	0.75
D5N	31 ± 7.2	8
P6S	170 ± 44	42.5
R7Nle	380 ± 81	95
R7K	295 ± 43	74
Peptide I	70 ± 9.8	17.5
Peptide II	3.7 ± 0.2	0.92

References

- [1] Betzel C., Lange G., Pal G.P., Wilson K.S., Maelicke A., Saenger W., The refined crystal structure of α -cobratoxin from *Naja naja siamensis* at 2.4 Å resolution, *J. Biol. Chem.* 266 (1991) 21530-21536.
- [2] Corringer P.J., Galzi J.L., Eiselé J.L., Bertrand S., Changeux J.P., Bertrand D., Identification of a new component of the agonist binding site of the nicotinic $\alpha 7$ homooligomeric receptor, *J. Biol. Chem.* 270 (1995) 11749-11752.
- [3] Drevet P., Lemaire C., Gasparini S., Zinn-Justin S., Lajeunesse E., Ducancel F., Pinkasfeld S., Courçon M., Trémeau O., Boulain J.C., Ménez A., High-level production and isotope labelling of snake neurotoxins, disulfide-rich proteins, *Prot. Exp. Purif.* 10 (1997) 293-300.
- [4] Ducancel F., Bouchier C., Tamiya T., Boulain J.C., Ménez A., Cloning and expression of cDNAs encoding snake toxins, in: Harvey A.L. (Ed.), *Snake Toxins*, Pergamon Press Inc., New York, 1991, 385-414.
- [5] Ducancel F., Mérienne K., Fromen-Romano C., Trémeau O., Pillet L., Drevet P., Zinn-Justin S., Boulain J.C., Ménez A., Mimicry between receptors and antibodies, *J. Biol. Chem.* 271 (1996) 31345-31353.
- [6] Endo T., Tamiya N., Structure-function relationships of post-synaptic neurotoxins from snake venoms, in: Harvey A.L. (Ed.), *Snake Toxins*, Pergamon Press Inc., New York, 1991, 165-222.
- [7] Fields C.G., Lloyd D.H., Macdonald R.L., Otteson K.M., Noble R.L., HBTU activation for automated Fmoc solid-phase peptide synthesis, *Peptide Res.* 4 (1991) 95-101.
- [8] Groebe D.R., Gray W.R., Abramson S.N., Determinants involved in the affinity of α -conotoxins GI and SI for the muscle subtype of nicotinic acetylcholine receptors, *Biochemistry* 36 (1997) 6469-6474.
- [9] Guddat L.W., Martin J.A., Shan L., Edmunson A.B., Gray W.R., Three-dimensional structure of the α -conotoxin GI at 1.2 Å resolution, *Biochemistry* 35 (1996) 11329-11335.
- [10] Hutchinson E.G., Thornton J.M., A revised set of potentials for β -turn formation in proteins, *Protein Sci.* 3 (1994) 2207-2216.
- [11] Johnson D.S., Martinez J., Elgoyhen A.B., Heinemann S.F., McIntosh J.M., α -Conotoxin ImI exhibits subtype-specific nicotinic acetylcholine receptor blockade: Preferential inhibition of homomeric $\alpha 7$ and $\alpha 9$ receptors, *Mol. Pharmacol.* 48 (1995) 194-199.
- [12] Lamthanh H., Virelizier H., Frayssinhes D., Side reaction of S-to-N acetamidomethyl shift during disulfide bond formation by iodine oxydation of S-acetamidomethyl-cysteine in a glutamine-containing peptide, *Pept. Res.* 8 (1995) 316-320.
- [13] McIntosh J.M., Yoshikami D., Mahe E., Nielsen D.B., Rivier J.E., Gray W.R., Olivera B.M., A nicotinic acetylcholine receptor ligand of unique specificity, α -conotoxin ImI, *J. Biol. Chem.* 269 (1994) 16733-16739.
- [14] Mérienne K., Germain N., Zinn-Justin S., Boulain J.C., Ducancel F., Ménez A., The functional architecture of an acetylcholine receptor-mimicking antibody, *J. Biol. Chem.* 272 (1997) 23775-23783.
- [15] Olivera B.M., Conus venom peptides, receptor and ion channel targets and drug design: 50 million years of neuropharmacology, *Mol. Biol. Cell* 8 (1997) 2101-2109.
- [16] Pereira E.F.R., Alkondon M., McIntosh J.M., Albuquerque E.X., α -Conotoxin ImI: A competitive antagonist at α -bungarotoxin-sensitive neuronal nicotinic receptors in hippocampal neurons, *J. Pharmacol. Exp. Ther.* 278 (1996) 1472-1483.
- [17] Romano S.J., Pugh P.C., McIntosh J.M., Berg D.K., Neuronal-type acetylcholine receptors and regulation of $\alpha 7$ gene expression in vertebrate skeletal muscle, *J. Neurobiol.* 32 (1997) 69-80.
- [18] Servent D., Winckler-Dietrich V., Hu H.Y., Kessler P., Drevet P., Bertrand D., Ménez A., Only snake curare-mimetic toxins with a fifth disulfide bond have high affinity for the neuronal $\alpha 7$ nicotinic receptor, *J. Biol. Chem.* 272 (1997) 24279-24286.
- [19] Sine S.M., Kneienkamp H.J., Bren N., Maeda R., Taylor P., Molecular dissection of subunit interfaces in the acetylcholine receptor: identification of determinants of α -conotoxin M1 selectivity, *Neuron* 15 (1995) 205-211.
- [20] Trémeau O., Lemaire C., Drevet P., Pinkasfeld S., Ducancel F., Boulain J.C., Ménez A., Genetic engineering of snake toxins, *J. Biol. Chem.* 270 (1995) 9362-9369.
- [21] Weber M., Changeux J.P., Binding of *Naja nigricollis* [3 H]- α toxin to membrane fragments from *Electrophorus* and *Torpedo* electric organs, *Mol. Pharmacol.* 10 (1974) 15-34.

Congenital myasthenic syndromes: Experiments of nature

Andrew G. Engel^a, Kinji Ohno^a, Steven M. Sine^b

^a*Department of Neurology and Neuromuscular Disease Laboratory and* ^b*Receptor Biology Laboratory, Department of Physiology and Biophysics, Mayo Clinic and Mayo Foundation, 200 First Street SW, Rochester, MN 55905, USA*

Abstract — Congenital myasthenic syndromes (CMS) can arise from presynaptic, synaptic, or postsynaptic defects. Recent studies indicate that mutations in the acetylcholine receptor (AChR) subunit genes are a common cause of the postsynaptic CMS. The mutations, which increase or decrease the response to acetylcholine, are experiments of nature that highlight functionally significant domains of the AChR. (©Elsevier, Paris)

Résumé — Les syndromes myasténiques congénitaux : Expériences de la nature. Les syndromes myasténiques congénitaux (SMC) peuvent être liés à des défauts présynaptiques, synaptiques ou postsynaptiques. Des études récentes indiquent que des mutations dans les gènes codant les sous-unités du récepteur à l'acétylcholine (RACH) sont une cause commune des SMC postsynaptiques. Les mutations, qui augmentent ou diminuent la réponse à l'acétylcholine, sont des expériences de la Nature qui rendent significatives les domaines fonctionnels de l'AChR. (©Elsevier, Paris)

congenital myasthenic syndromes / acetylcholine receptor / mutation analysis / expression studies

1. Introduction

Congenital myasthenic syndromes (CMS) are heterogeneous disorders arising from presynaptic, synaptic, or postsynaptic defects. In each CMS, the specific defect compromises the safety margin of neuromuscular transmission by one or more mechanisms. The clinical phenotypes of CMS are often similar; therefore, precise diagnosis requires correlation of clinical, in vitro electrophysiological, morphological, and, whenever possible, molecular genetic studies [3].

Prior to 1990, the investigation of CMS patients was based on clinical, morphologic, and conventional microelectrode studies. Since then, three developments paved the way for molecular analysis of postsynaptic CMS. First, by 1993, the cDNA sequences of the α , β , δ and ϵ subunits of adult and of the γ subunit of fetal human AChR were known, allowing molecular genetic analysis. Second, in the early 1990s, Milone et al. [10] succeeded in patch-clamping endplates (EPs) in human intercostal muscles, permitting analysis of the activity of single AChR channels. Third, the use of mammalian expression systems facilitated detailed analysis of how human AChR mutants alter the kinetics of the AChR channel. Coincident with this, we hypothesized that a kinetic abnormality of AChR at the single channel level predicts, and that severe EP AChR deficiency may predict, one or more mutations in the subunits of AChR. This hypothesis was subsequently confirmed by the discovery of mutations in different subunits of AChR that either increase [5, 13, 18, 21,

23] or decrease [4, 12, 14–17, 21, 22] the synaptic response to ACh.

2. Mutations in AChR subunits cause postsynaptic CMS

Since 1994, we and other investigators identified 56 AChR subunit gene mutations in 69 CMS kinships. *Table 1* indicates the identified mutations according to their functional consequences and subunit locations. Interestingly, 38 of the 56 mutations and all 27 null mutations occur in the ϵ subunit of AChR, highlighting the susceptibility of the gene to mutation.

3. Increased response to ACh: slow-channel mutations

The clues for the diagnosis of an SCCMS consist of selectively severe weakness of the forearm extensor muscles, a repetitive compound muscle action potential response to single nerve stimuli that is accentuated by edrophonium, and a prolonged and biexponentially decaying miniature EP current. Eleven SCCMS mutations have been reported to date [2, 5, 7, 13, 18–20, 23, 25]. The different mutations occur in different AChR subunits and in different functional domains of the subunits (*figure 1A*). Each is dominant, causing a pathologic gain of function.

The phenotypic consequences of the SCCMS mutations stem from prolonged opening episodes of the

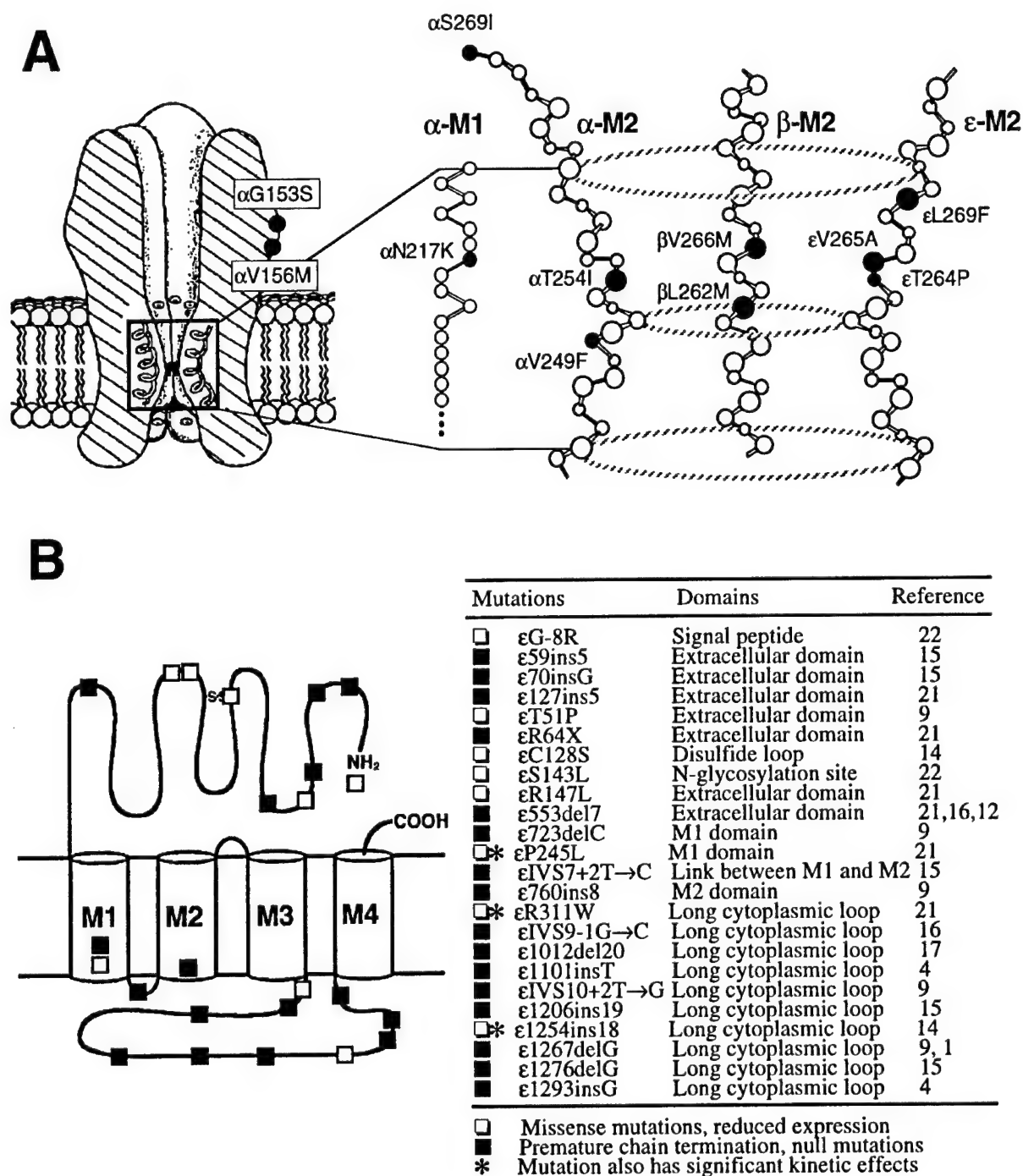


Figure 1. A. Schematic diagrams of slow-channel CMS mutations reported to date. Cartoon on the left shows a section through AChR lodged in a lipid bilayer. The extracellular part of AChR forms a funnel-shaped channel that narrows to a gate formed by the alpha-helical M2 domain of each subunit. Two α subunit mutations in the extracellular domain near the ACh binding site are indicated. Cartoon on the right shows slow-channel mutations detected between the M2 and M3 domains of the α subunit, in the M2 domains of the α , β and ϵ subunits, and in the M1 domain of the α subunit. B. Schematic diagram and list of 24 low-expressor or null mutations in the ϵ subunit.

Table I. Fifty-six AChR subunit gene mutations in 69 kinships.

Mutation	α	β	δ	ϵ	Total
<i>Point mutations</i>					
Slow channel mutation	8	2		3	13
Fast channel mutation	1			1	2
Null mutation				2*	2
Reduced expression	4		2	5	11
<i>Inframe rearrangement</i>		1		2	3
<i>Premature chain termination</i> (null mutations)					
Frameshifting rearrangement				16*	16
Splice-site mutation				6*	6
Nonsense mutation				3*	3
Total	13	3	2	38	56

Includes 34 published and 17 unpublished CMS mutations observed in our laboratories, three slow-channel mutations in the α subunit [2] and a frameshifting rearrangement in the ϵ subunit [1] described by Croxen et al., and a slow-channel mutation in the β subunit detected by Gomez et al. [7]. Numbers in bold face indicate recessive mutations that reduce AChR expression.

* Null mutations.

AChR channel. These cause: 1) cationic overloading of the junctional sarcoplasm and an EP myopathy with loss of AChR from degenerating junctional folds; and 2) a depolarization block due to staircase summation of prolonged end-plate potentials [5, 18, 23]. Patch clamp studies at the EP, mutation analysis, and expression studies in human embryonic kidney fibroblast (HEK) cells dissected three types of SCCMS. Those residing in the M2 domain, which lines the channel pore, act predominantly by slowing channel closure [5, 18]. A mutation near the ACh binding site on the α subunit increases affinity of AChR for ACh causing repeated reopenings of the channel during the prolonged ACh occupancy [23]. Another type of CMS has features of the two preceding types and the mutations reside in the M1 or M2 domain [5, 13, 25].

Recent studies indicate that quinidine, a long-lived open-channel blocker of AChR, is beneficial in the SCCMS. Fukudome et al. [6] demonstrated that drug levels attainable in clinical practice shorten and even normalize the prolonged opening episodes of mutant SCCMS AChRs expressed in HEK cells, and Harper and Engel [8] found that doses of the drug producing serum levels of 0.7-2.5 $\mu\text{g/mL}$ (2.1-7.7 $\mu\text{M/L}$) benefit SCCMS patients by clinical and EMG criteria.

4. Decreased response to ACh: the low-affinity fast-channel mutations

Mutation analysis in two patients revealed two mutations in different alleles of the ϵ subunit gene:

a common ϵP121L mutation plus a null mutation in the second ϵ allele, so that ϵP121L defined the clinical phenotype. In these patients the postsynaptic response to ACh is markedly diminished although the number of AChR per EP is normal [22, 24]. Patch-clamp studies show infrequent AChR channel events, abnormally brief activation episodes due to diminished channel reopenings during ACh occupancy, and increased resistance to desensitization by ACh [22]. Genetically engineered ϵP121L -AChR expressed in HEK cells have markedly decreased rate of channel opening and show greatly reduced affinity for ACh in the open-channel and desensitized states [22].

It is interesting to note that the ϵP121L mutation and the SCCMS mutations have opposite effects: SCCMS mutations increase the duration of activation episodes, enhance ACh binding affinity, increase desensitization by ACh, and cause an EP myopathy; ϵP121L shortens the duration of activation episodes, reduces ACh binding affinity, decreases desensitization by ACh, and leaves no anatomic footprint.

Recently we encountered a second low-affinity fast-channel mutation, αV285I , combined with a low-expressor mutation, αF233V , in the other α allele [11]. Detailed kinetic studies of αV285I are in progress.

5. Decreased response to ACh: a mutation causing mode-switching kinetics

In this disorder, an inframe duplication in the long cytoplasmic loop of ϵ , $\epsilon\text{1254ins18}$, appears in com-

bination with a cysteine-loop null mutation, ϵ C128S [14]. The ϵ 1254ins18 mutation, which determines the phenotype, causes mode switching in the kinetics of receptor activation in which the normal high efficiency of gating is accompanied by two new modes that gait inefficiently. In the two abnormal modes the channel opens more slowly and closes more rapidly than normal. The ϵ 1245ins18 AChR at the endplate shows abnormally brief activation episodes during steady state agonist application, and appears electrically silent during the synaptic response to acetylcholine. The phenotypic consequences are endplate AChR deficiency, simplification of the postsynaptic region, and compensatory expression of fetal AChR that restores electrical activity at the endplate and rescues the phenotype.

6. AChR deficiency caused by recessive mutations in AChR subunits

Severe EP AChR deficiency can result from different types of recessive mutations in AChR subunit genes. The mutations are either homozygous or, more frequently, heterozygous. Morphologic studies show an increased number of EP regions distributed over an increased span of the muscle fiber. The integrity of the junctional folds is preserved but some EP regions are simplified and smaller than normal. The distribution of AChR on the junctional folds is patchy and the density of the reaction for AChR is attenuated. Conventional microelectrode studies reveal a decreased amplitude of the miniature EP potentials and currents, and frequently high or higher than normal quantal release by nerve impulse. Single channel recordings at the EP [12, 14, 21] or immunocytochemical studies [4] often reveal the presence of fetal AChR that harbors the γ instead of the adult ϵ subunit (γ -AChR) at the EP.

Different types of recessive mutations causing severe EP AChR deficiency have now been identified (*figure 1B*): 1) mutations causing premature termination of the translational chain. These mutations are frameshifting [4, 9, 15, 17, 21], occur at a splice site [9, 15, 16], or produce a stop codon directly [21]; 2) missense mutation in a signal peptide region (ϵ G-8R) [22]; 3) missense mutations in residues essential for assembly of the pentameric receptor. Mutations of this type were observed in the ϵ subunit at an N-glycosylation site (ϵ S143L) [22]; in cysteine 128 (ϵ C128S), a residue that is an essential part of the C128-C142 disulfide loop in the extracellular domain [14]; and in arginine 147 (ϵ R147L) in the extracellular domain, which lies between isoleucine 145 and threonine 150, residues that contribute to subunit assembly [21]; and 4) missense mutations

affecting both AChR expression and kinetics. For example, ϵ R311W [21] in the long cytoplasmic loop between M3 and M4 decreases, whereas ϵ P245L in the M1 domain [21] increases the open duration of channel events. In the case of ϵ R311W and ϵ P245L, the kinetic consequences are modest and are likely overshadowed by the reduced expression of the mutant gene.

There are two possible reasons for why recessive mutations causing AChR deficiency are concentrated in the ϵ subunit. First, expression of the fetal type γ subunit, although at a low level, may compensate for absence of the ϵ subunit [4, 14, 21], whereas patients harboring null mutations in subunits other than ϵ might not survive for lack of a substituting subunit. Second, the gene encoding the ϵ subunit, and especially the exons coding for the long cytoplasmic loop, have a high GC content that likely predisposes to DNA rearrangements.

7. Future prospects

Since 1994, molecular analysis of the postsynaptic CMS provided clear insights into disease mechanisms and highlighted functionally significant domains of AChR. In the coming years molecular studies will undoubtedly be applied to presynaptic and synaptic forms of CMS. It is also likely that the molecular studies will provide clues for conventional and gene therapy and lead to the recognition of novel types of CMS.

Acknowledgments

Work in the authors' laboratories was supported NS6277 (AGE) and NS31744 (SMS) from the National Institutes of Health, Bethesda, MD, and a research grant from the Muscular Dystrophy Association (AGE).

References

- [1] Croxen R., Beeson D., Vincent A., et al., Congenital myasthenic syndrome with a single nucleotide deletion at the intron/exon boundary in exon 12 of the gene encoding the acetylcholine receptor ϵ subunit, (abstract), *Ann. Neurol.* 40 (1996)513.
- [2] Croxen R., Newland C., Beeson D., Oosterhuis H., Chauplanaz G., Vincent A., Newsom-Davis J., Mutations in different functional domains of the human muscle acetylcholine receptor α subunit in patients with the slow-channel congenital myasthenic syndrome, *Hum. Mol. Genet.* 6 (1997) 767-773.
- [3] Engel A.G., Myasthenic syndromes, in: Engel A.G., Franzini-Armstrong C., (Eds.), 2 edn., *Myology: Basic and Clinical*, McGraw-Hill, New York, 1994, pp. 1798-1835.

- [4] Engel A.G., Ohno K., Bouzat C., Sine S.M., Griggs R.G., End-plate acetylcholine receptor deficiency due to nonsense mutations in the ϵ subunit, *Ann. Neurol.* 40 (1996) 810–817.
- [5] Engel A.G., Ohno K., Milone M., Wang H.-L., Nakano S., Bouzat C., Pruitt J.N., Hutchinson D.O., Brengman J.M., Bren N., Sieb J.P., Sine S.M., New mutations in acetylcholine receptor subunit genes reveal heterogeneity in the slow-channel congenital myasthenic syndrome, *Hum. Mol. Genet.* 5 (1996) 1217–1227.
- [6] Fukudome T., Ohno K., Brengman J.M., Engel A.G., Quinidine normalizes the open duration of slow-channel mutants of the acetylcholine receptor, *NeuroReport* 9 (1998) 1907–1911.
- [7] Gomez C.M., Maselli R., Gammack J., Lasalde J., Tamamizu S., Cornblath D.R., Lehar M., McNamee M., Kunkel R., A beta-subunit mutation in the acetylcholine receptor gate causes severe slow-channel syndrome, *Ann. Neurol.* 39 (1996) 712–723.
- [8] Harper C.M., Engel A.G., Quinidine sulfate therapy for the slow-channel congenital myasthenic syndrome, *Ann. Neurol.* 43 (1998) 480–484.
- [9] Middleton L., Ohno K., Christodoulou K., et al., Congenital myasthenic syndromes linked to chromosome 17p are caused by defects in acetylcholine receptor ϵ subunit gene (abstract), *Neurology* 50 (1998) A432.
- [10] Milone M., Hutchinson D.O., Engel A.G., Patch-clamp analysis of the properties of acetylcholine receptor channels at the normal human endplate, *Muscle Nerve* 17 (1994) 1364–1369.
- [11] Milone M., Ohno K., Brengman J.M. et al., Low-affinity fast-channel congenital myasthenic syndrome caused by new missense mutations in the acetylcholine receptor α subunit (abstract), *Neurology* 50 (1998) A432–A433.
- [12] Milone M., Ohno K., Pruitt J.N., Brengman J.M., Sine S.M., Engel A.G., Congenital myasthenic syndrome due to frameshifting acetylcholine receptor epsilon subunit mutation, *Soc. Neurosci. Abstr.* 22 (1996) 1942–1942.
- [13] Milone M., Wang H.-L., Ohno K., Fukudome K., Pruitt J.N., Bren N., Sine S.M., Engel A.G., Slow-channel syndrome caused by enhanced activation, desensitization, and agonist binding affinity due to mutation in the M2 domain of the acetylcholine receptor alpha subunit, *J. Neurosci.* 17 (1997) 5651–5665.
- [14] Milone M., Wang H.-L., Ohno K., Prince R.J., Shen X.-M., Brengman J.M., Griggs R.C., Engel A.G., Mode switching kinetics produced by a naturally occurring mutation in the cytoplasmic loop of the human acetylcholine receptor ϵ subunit, *Neuron* 20 (1998) 575–588.
- [15] Ohno K., Anlar B., Özdirim E., Brengman J.M., De Bleecker J., Engel A.G., Myasthenic syndromes in Turkish kinships due to mutations in the acetylcholine receptor, *Ann. Neurol.* (1998), in press.
- [16] Ohno K., Engel A.G., Milone M., et al., A congenital myasthenic syndrome with severe acetylcholine receptor deficiency caused by heteroallelic frameshifting mutations in the epsilon subunit (abstract), *Neurology* 45 (1995) (suppl. 4) A283.
- [17] Ohno K., Fukudome T., Nakano S., Milone M., Feasby T.E., Tyce G.M., Engel A.G., Mutational analysis in a congenital myasthenic syndrome reveals a novel acetylcholine receptor epsilon subunit mutation, *Soc. Neurosci. Abstr.* 22 (1996) 234–234.
- [18] Ohno K., Hutchinson D.O., Milone M., Brengman J.M., Bouzat C., Sine S.M., Engel A.G., Congenital myasthenic syndrome caused by prolonged acetylcholine receptor channel openings due to a mutation in the M2 domain of the ϵ subunit, *Proc. Natl. Acad. Sci. USA.* 92 (1995) 758–762.
- [19] Ohno K., Hutchinson D.O., Milone M., et al., Molecular genetic basis of a slow channel syndrome, (abstract), *Muscle Nerve* 18 (1995) 463.
- [20] Ohno K., Milone M., Brengman J.M., et al., Slow-channel congenital myasthenic syndrome caused by a novel mutation in the acetylcholine receptor ϵ subunit (abstract), *Neurology* 50 (1998) A432.
- [21] Ohno K., Quiram P., Milone M., Wang H.-L., Harper C.M., Pruitt J.N., Brengman J.M., Pao L., Fischbeck K.H., Crawford T.O., Sine S.M., Engel A.G., Congenital myasthenic syndromes due to heteroallelic nonsense/missense mutations in the acetylcholine receptor ϵ subunit gene: identification and functional characterization of six new mutations, *Hum. Mol. Genet.* 6 (1997) 753–766.
- [22] Ohno K., Wang H.-L., Milone M., Bren N., Brengman J.M., Nakano S., Quiram P., Pruitt J.N., Sine S.M., Engel A.G., Congenital myasthenic syndrome caused by decreased agonist binding affinity due to a mutation in the acetylcholine receptor ϵ subunit, *Neuron* 17 (1996) 157–170.
- [23] Sine S.M., Ohno K., Bouzat C., Auerbach A., Milone M., Pruitt J.N., Engel A.G., Mutation of the acetylcholine receptor α subunit causes a slow-channel myasthenic syndrome by enhancing agonist binding affinity, *Neuron* 15 (1995) 229–239.
- [24] Uchitel O., Engel A.G., Walls T.J., Nagel A., Atassi Z.M., Brill V., Congenital myasthenic syndromes. II. A syndrome attributed to abnormal interaction of acetylcholine with its receptor, *Muscle Nerve* 16 (1993) 1293–1301.
- [25] Wang H.-L., Auerbach A., Bren N., Ohno K., Engel A.G., Sine S.M., Mutation in the M1 domain of the acetylcholine receptor α subunit decreases the rate of agonist dissociation, *J. Gen. Physiol.* 109 (1997) 757–766.

Regulated exocytosis in neurons and neurosecretory cells: Structural events and expression competence

Jacopo Meldolesi*

Department of Pharmacology and B.-Ceccarelli Center, University of Milan; CNR Center of Molecular and Cellular Pharmacology and DIBIT, Department of Neurosciences, S.-Raffaele Institute, via Olgettina, 58, 20132 Milan, Italy

Abstract — This paper summarizes the contribution of the laboratory first in the development of the 'kiss-and-run model' of exocytosis, with its fascinating aspects of specificity and rapidity, then in proposing the existence of 'competence factors' that appear to govern the appearance of the secretory vesicles and exocytic process in neurosecretory cells. (©Elsevier, Paris)

Résumé — **Exocytose contrôlée dans les neurones et les cellules neurosécrétrices : événements structuraux et compétence cellulaire.** Cet article résume les contributions de notre laboratoire, d'une part dans le développement du modèle d'exocytose 'kiss-and-run', avec ses aspects fascinants de spécificité et de rapidité, et d'autre part dans l'hypothèse de l'existence de « facteurs de compétence » qui semblent contrôler la formation de vésicules de sécrétion et les processus d'exocytose dans les cellules neurosécrétrices. (©Elsevier, Paris)

regulated exocytosis / vesicle fusion / vesicle recycling / neurosecretion competence

1. Introduction

Exocytosis in neurons and neurosecretory cells, i.e., the process by which quanta of neurotransmitter are released in bulk and made available to the specific receptors in the surrounding membranes, has been a major interest of our group. Studies were initiated by Bruno Ceccarelli in the early seventies and later developed in multiple directions by his and my laboratory. Soon after the exocytic process was identified, it became clear that the cellular aspects are more complex and elaborate than initially thought inasmuch as the step of vesicle fusion is rapidly followed by the recycling of the fused membrane, necessary to maintain the plasmalemma specificity in terms of both surface extension and molecular composition. The nomenclature of exo-endocytosis (or exo-endocytic cycle) was thus proposed, and attention was focused more and more on the molecular mechanisms and on their regulation, with progressive identification of the proteins involved, both in the membranes and in the cytosol. Surprisingly, however, for quite sometime the structural features and the time-course of the cycle did attract only few specific studies. Even at synapses the recycling of fused vesicles was largely believed to occur by a process of conventional endocytosis: formation of a coated

cage around the plasmalemmal invagination resulting from an exocytic fusion, detachment of the resulting coated vesicle from the plasmalemma, shedding of the coat followed by fusion of the naked vesicle with a cisterna of the endocytic compartment from which new synaptic vesicles were believed to originate by budding (for a classical discussion of the problem see Ceccarelli and Hurlbut [2]). Such a sequence of events, although necessarily slow in its development, was so successful that it is still the only one reported in most textbooks, even in some of the most advanced.

Among the few scientists that remained not convinced by the 'coated vesicle model' were those of our group. For quite sometime the success of alternative explanations remained limited. Yet, intriguing suggestions were offered even by plain electron microscopy, when applied however not to conventionally fixed samples but to preparations that had been quick frozen and thus blocked within 1 ms in the course of their stimulated exocytic process. With the latter approach many of the vesicles more intimately continuous with the plasmalemma appear in fact not as invaginations, open to the extracellular space, but still as vesicles, sealed however by thin, clearly discernible diaphragms in direct continuity with the cell surface. The interpretation that Ceccarelli and his colleagues provided to the images was that of transient and incomplete fusions, adequate for the release of the neurotransmitter quanta. Such a mechanism can assure the rapid recycling of the vesicles, with

* Correspondence and reprints to via Olgettina, 58, 20132 Milan.

no risk of molecular membrane intermixing with the plasmalemma [2, 7]. In our more recent review, written a few years after Bruno Ceccarelli's death, this exo-endocytic process was first referred to as 'kiss-and-run' [4], a definition that is now encountering favor in the scientific community. The favor is not only due to the suggestive power of the definition. Rather, new and independent evidence has accumulated that appears at least compatible with the 'kiss-and-run' model of exocytosis. In fact, very transient openings of secretory organelles do indeed take place in at least some secretory systems, as revealed by capacitance measurements in patch clamping [1, 5]; exocytosis can be dissociated from recycling of membrane invaginations [6]; interactions of recycled vesicles with endosomes have now been excluded [8].

To sum up, the exo-endocytic field appears now more open than a few years ago (see also Schweitzer et al. [9]). Kiss-and-run can be envisaged as one (the most convenient for the cell!) of the processes by which exo-endocytosis can take place. When in contrast the opening of a fused vesicle proceeds from the transient to a more persistent stage, possibly because of the disassembly of an initial, channel-like structure around the vesicle opening [5], recycling requires more complex events involving the contractile protein, dynamin together with clathrin and its coats [10]. Whatever the frequency of the two processes (and possibly of others that remain to be identified), the lesson for future work is to pay attention to properties, such as timing and number of vesicles involved, that in most studies carried out until recently had been let aside, with ensuing missing of important aspects of the exo-endocytic process.

2. Regulated exocytosis: what about its cell expression?

Regulated exocytosis with release of specific products is widespread, but not ubiquitous among eukaryotic cell types. The question then is: how do cells acquire the ability to assemble specialized organelles (dense and clear vesicles in the case of neurons and neurosecretory cells) together with the molecular apparatus (not only in vesicles but also in the plasmalemma and in the cytosol) that, in response to appropriate stimulation, governs exocytosis followed by recycling? Since in the appropriate cells the appearance of vesicles of either type takes place during embryonic development, exocytosis is often thought to represent a step in phenotype acquisition. However, results that we have obtained during the last several years with a clone of PC12, a cell line derived from a rat pheochromocytoma, suggest the sto-

ry to be more complex. Our clone, in fact, was found to lack completely the secretory organelles together with their membrane and cargo components, and to be also free of the specific cytosolic and plasmalemma proteins participating in regulated exocytosis. Yet, the clone is not de-differentiated with respect to the other PC12 cells. In contrast, it does express a variety of specific markers, thus its cells can only be defined as neurosecretory but defective of regulated neurosecretion competence [3].

The latter conclusion suggests that regulated exocytosis is a property expressed independently from the others of the neurosecretory cell phenotype, governed by specific factor(s) that operate 'in block', by switching on (or off) the expression of multiple and variously targeted components of the cell. The nature of the 'secretion competence factor(s)', although intensively investigated during the last few years, is at the moment still mysterious. Evidence of its existence has however been obtained by cell fusion experiments. Reappearance of neurosecretion, specific in molecular terms, has been observed in chimeras obtained by the fusion of defective PC12 not only with secretory but also with non-secretory cells (unpublished results). A new, so far unexplored line of exocytosis research, concerning the cell competence and expression of the process, has thus been initiated. Although at the moment its success is hard to predict, the study appears nevertheless fascinating, also because it necessarily requires the combination of multiple advanced experimental approaches, focused on a single, well defined and original issue. Let's wait for the developments.

Acknowledgments

I wish to express my gratitude to the numerous colleagues involved in the summarized experiments, identified in the references of the laboratory. Support for part of the work was obtained from the E.U. Biotech Program and from the Human Frontier Science Program.

References

- [1] Artalejo C.R., Elhamdani A., Palfrey H.C., Secretion: dense-core vesicles can kiss-and-run too, *Curr. Biol.* 8 (1998) R62-R65.
- [2] Ceccarelli B., Hurlbut W.P., Vesicle hypothesis of the release of quanta of acetylcholine, *Physiol. Rev.* 60 (1980) 396-441.
- [3] Corradi N., Borgonovo B., Clementi E., Bassetti M., Racchetti G., Consalez G.G., Huttner W.B., Meldolesi J., Rosa P., Overall lack of regulated secretion in a PC12 variant cell clone, *J. Biol. Chem.* 271 (1996) 27116-27124.
- [4] Fesce R., Grohovaz F., Valtorta F., Meldolesi J., Neurotransmitter release: fusion or kiss-and-run?, *Trends Cell Biol.* 4 (1994) 1-4.

- [5] Henkel A.W., Almers W., Fast steps in exocytosis and endocytosis studied by capacitance methods in endocrine cells, *Curr. Opin. Neurobiol.* 6 (1996) 350-357.
- [6] Henkel A.W., Betz W.J., Staurosporine blocks evoked release of FM1-43 but not acetylcholine from frog motor nerve terminals, *J. Neurosci.* 15 (1995) 8255-8257.
- [7] Meldolesi J., Ceccarelli B., Exocytosis and membrane recycling, *Phil. Trans. R. Soc. Lond. B.* 296 (1981) 55-65.
- [8] Murthy V.N., Stevens C.F., Synaptic vesicles retain their identity through the endocytic cycle, *Nature* 393 (1998) 497-501.
- [9] Schweizer F.E., Betz H., Augustine G.J., From vesicle docking to endocytosis: intermediate reactions of exocytosis, *Neuron* 14 (1995) 689-696.
- [10] Takei K., Mundigl O., Daniell L., De Camilli P., The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin, *J. Cell Biol.* 133 (1996) 1237-1250.

Acetylcholine release. Reconstitution of the elementary quantal mechanism

Maurice Israël^a, Yves Dunant^b

^aLaboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, 91198 Gif-sur-Yvette, France

^bDépartement de Pharmacologie, CMU 1211 Geneva, Switzerland

Abstract — Choline acetyltransferase and vesicular acetylcholine transporter genes are the products of two adjacent genes defining a cholinergic locus. The release mechanism is expressed independently of this locus in some cell lines. A cholinergic neuron will therefore have to coordinate the expression of release with that of the cholinergic locus. Transfection of a plasmid encoding *Torpedo* mediatoaphore in cells that are unable to release this transmitter endows them with a Ca^{2+} -dependent and quantal release mechanism. The synchronization of mediatoaphore activation results from a control of calcium microdomains by the synaptic vesicles. It is therefore dependent on the proteins that dock vesicles close to calcium channels. (©Elsevier, Paris)

Résumé — Libération d'acétylcholine. Reconstitution du mécanisme quantique élémentaire. La choline acétylase et le transporteur vésiculaire d'acétylcholine sont codés par deux gènes adjacents qui définissent un locus cholinergique. Certaines lignées cellulaires expriment le mécanisme de libération de l'acétylcholine indépendamment de ce locus cholinergique. Un neurone cholinergique devra donc coordonner l'expression du locus cholinergique et celle du mécanisme de libération. La transfection de cellules incapables de libérer l'acétylcholine avec un plasmide codant pour le médiatoaphore de Torpille leur confère un mécanisme de libération Ca^{2+} -dépendant et quantique. La synchronisation des médiatoaphores est liée au contrôle de microdomaines calciques par les vésicules synaptiques. Elle dépend donc des protéines qui assurent l'ancrage des vésicules à proximité des canaux calciques. (©Elsevier, Paris)

acetylcholine release / cholinergic locus / mediatoaphore / transfection

1. Introduction

It has long been known that proteins supporting cholinergic transmission are expressed in a variety of cells that are not directly involved in synaptic transmission. For example, acetylcholinesterase is present in erythrocytes [1], choline-acetyltransferase in the placenta [34] and nicotinic receptors at the tendon [25]. Unexpected localization of the release machinery itself has also been suspected from recent experiments showing that glial cells, fibroblasts and other cell types, loaded in culture with acetylcholine (ACh), are subsequently able to release the neurotransmitter, either in response to a calcium influx or after electrical stimulation [11–13, 20]. Also fibroblasts transfected with choline-acetyltransferase cDNA become able to release the synthesized transmitter [14] and myocytes injected with ACh can generate miniature end-plate potentials [9]. More recently, both spontaneous and evoked ACh release were demonstrated in fibroblast cell lines [16, 27]. Moreover, proteins that are typical for the active zone of nerve terminals belong to protein families whose other members are found even in yeast, suggesting a very broad role in cellular communication [31].

2. Results and discussion

2.1. Unexpected expression of the release mechanism in different cell lines

The localization of choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VACHT) genes within a single genomic locus and the co-expression of both proteins throughout the cholinergic system strongly suggests that synthesis and storage of ACh are tightly co-regulated [2, 4, 10, 26]. But what about regulation of the release mechanism? A variety of cell lines that do not express the ChAT and VACHT genes nonetheless display a Ca^{2+} -dependent quantal release mechanism for ACh, as discovered after loading the cells in culture with ACh [12, 20] or after transfection with ChAT cDNA [14, 21]. Taken together, these data demonstrate that the proteins involved in release can be expressed independently of ChAT or VACHT.

Ca^{2+} -dependent ACh release was monitored from four different cell lines by using the choline-oxidase procedure. Cells were loaded with ACh during culture [20] and release was elicited with a calcium ionophore (A23187), followed by calcium. In these experiments, the amount of ACh was the same in

the different cell samples. It was measured before the release challenge and the sample size adjusted accordingly. As seen in figure 1 (top traces), cells from the glioma C6BU-1, the fibroblast L-M (TK-) and the hybrid NG108-15 lines were found to possess a release mechanism, whereas neuroblastoma N18TG-2 cells were unable to release the transmitter. The neuroblastoma-glioma NG108-15 hybrid line (which is issued from C6BU-1 and N18TG-2 lines) might therefore have 'inherited' the release machinery from its glial parent. The pattern of release-competent and release-incompetent cells presented here was a constant finding with these cell lines.

An electrophysiological approach enabled us to characterize the release mechanism with a more adequate time resolution. The results were in full agreement with those of the biochemical assay (figure 1, bottom traces). ACh-filled cells were individually put into contact with a *Xenopus* myoball that served as a

real-time ACh detector. On electrical stimulation of C6BU-1, L-M (TK-) and NG108-15 cells, rapid inward currents were recorded from the patched myoball. These 'postjunctional' currents were reversibly suppressed by addition of tubocurarine or when calcium was replaced by 10 mM magnesium in the solution. In series of responses generated by a given cell, the evoked currents showed a tendency to peak at preferential steps, suggesting a quantal composition. In contrast to the other lines, N18TG-2 cells did not express stimulation-evoked ACh release. No response, or only responses of a negligible amplitude, were observed after testing more than 130 ACh-filled N18TG-2 cells.

2.2. Acetylcholine release from mediatophore-transfected cells

Mediatophore is a protein we had characterized in previous works. Purified from the plasma mem-

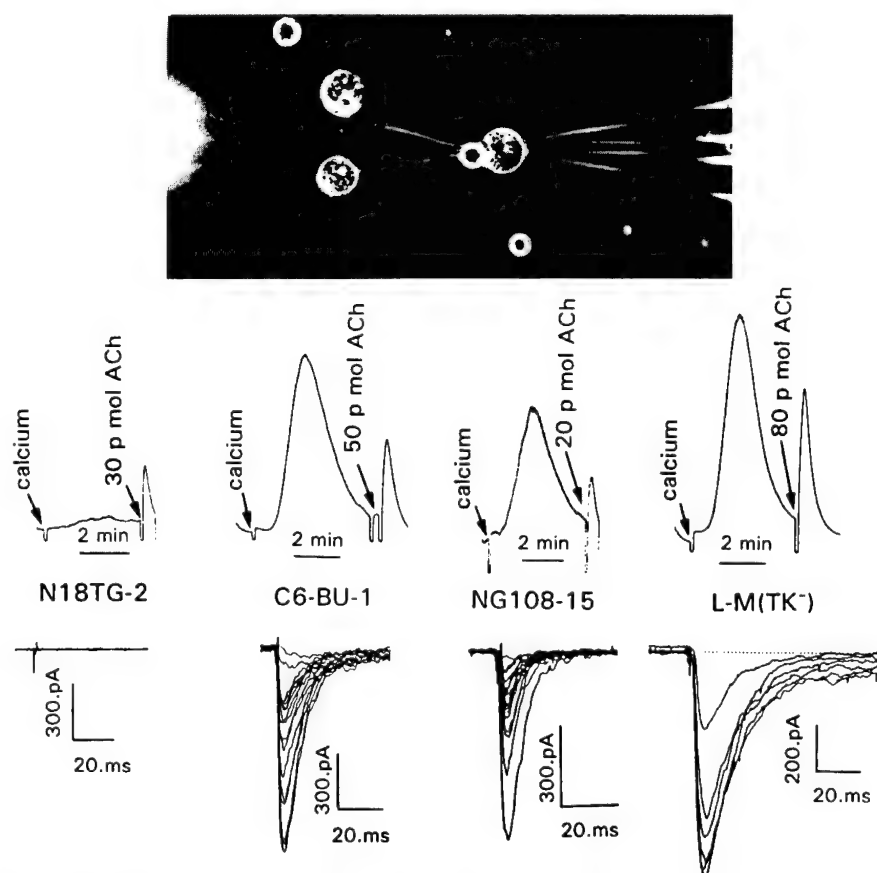


Figure 1. ACh release elicited from various cell lines loaded with the transmitter. Top traces: release was triggered by calcium after treatment with ionophore A23187 and measured continuously with the luminescence assay. Bottom traces: release from the same cell lines, but in this case release was evoked by electrical stimulation and measured in real-time by using a patched *Xenopus* myocyte. Top picture: a releasing cell (on the left) in contact with the *Xenopus* myocyte. The neuroblastoma N18TG-2 cells were not release-competent, while the rat glioma C6-Bu-1, the glioma-neuroblastoma hybrid NG108-15 and the fibroblast L-M (TX) lines expressed Ca^{2+} -dependent and quantal transmitter release.

brane of *Torpedo* electric organ synaptosomes, mediator is able to translocate ACh upon calcium action [19, 20]. Mediator is localized at the active zone of nerve terminals [6]. It is a homo-oligomer of a 16-kDa subunit that is also found in association with other components in the membrane sector of the V-ATPase [35]. Antisense probes hybridizing the mediator messenger inhibit ACh release [7, 8, 20].

We have recently shown that cells with a low 16-kDa proteolipid content in their membrane have a low capacity for ACh release. This was the case for N18GT-2 cells. They consequently represented an ideal material for a reconstitution of ACh release. N18GT-2 cells were transfected with a plasmid en-

coding the *Torpedo* mediator. The Western blot in figure 2 demonstrates that the transfected cells express the *Torpedo* protein. Several clones were selected and tested for release. The N18GT-2 cells that expressed *Torpedo* mediator gained the capability to release ACh in response to a calcium influx (figure 2, top traces). Control clones, transfected with the plasmid alone, did not release the transmitter although they contained the same amount of ACh.

Control and transfected cells were also tested for release by using patched *Xenopus* myoballs. Stimulation elicited a pulse-like release of transmitter from mediator-transfected cells, but not from controls (figure 2, bottom traces). In series of successive responses, as those illustrated in figure 2, the currents

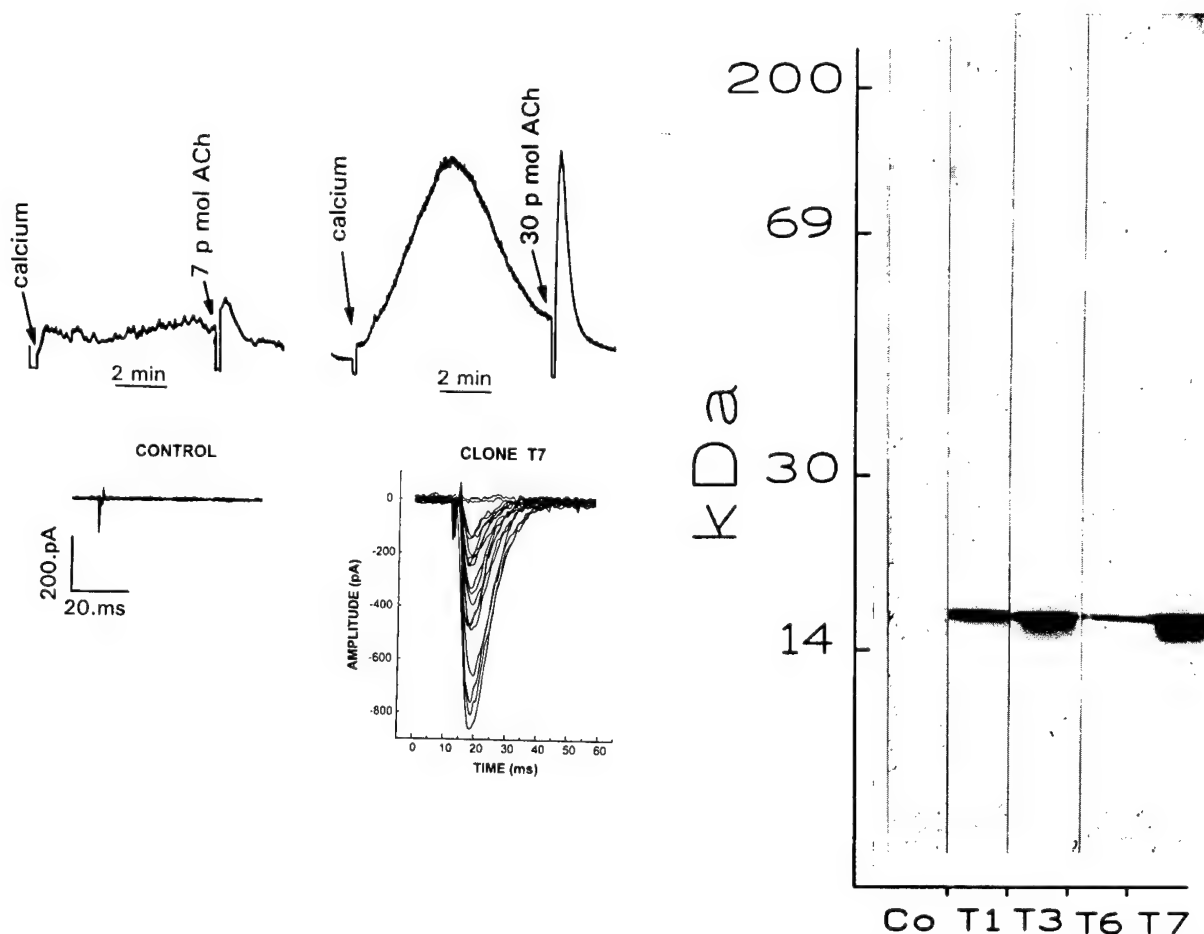


Figure 2. Right part: Western blot showing that *Torpedo* mediator was expressed in several clones (T1 to T7) of N18GT-2 cells after transfection. 'Co' is a control clone containing the transfection vector without the mediator insert. The control cells did not release ACh, while the mediator-transfected clones acquired a new release capacity. Release was measured by the choline oxidase chemiluminescent procedure (top traces) and recorded electrophysiologically by using a patched myoball (bottom traces). Transfection with the 16-kDa subunit of the mediator re-established a ACh release which was typically Ca^{2+} -dependent and quantal, like in natural synapses.

peaked at preferential amplitude values, suggesting a quantal composition of transmitter release. This was further demonstrated by analyzing the successive responses using the Poisson's distribution [11]. As with the cell lines described above, it was verified that evoked ACh release induced by transfection in N18TG-2 cells was curare-sensitive and Ca^{2+} -dependent. Hence, release-deficient cells which had acquired by transfection the *Torpedo* mediato-phore in their plasma membrane became able to release ACh in a pulse-like and quantal form, which is the most characteristic property of transmitter release in naturally-occurring synapses.

2.3. A common regulation is required to turn on both the cholinergic locus and the release mechanism

In spite of the fact that the cholinergic release mechanism can be expressed independently of synthesis and storage, cholinergic neurons will have to turn on both the release apparatus and the cholinergic locus by some coordinated regulation. For the release, the problem is complex since many proteins might be involved. Nevertheless, the approach can be simplified by separately considering those proteins that are more specifically related to the release of ACh and those that are common to many cells. The latter include the components of the T- and V-SNAREs [28] that govern the traffic of intracellular membranes at the active zone as they do in the Golgi apparatus. These proteins (the T-SNAREs syntaxin and SNAP-25 at the plasma membrane, and the V-SNARE VAMP/synaptobrevin on synaptic vesicles) are expressed in many cell types. The same is true for V-ATPase, which functions to accumulate protons in intracellular organelles. In neurons, it is this process which is responsible for the driving force concentrating the transmitters into granules or vesicles.

The protein supporting more specifically ACh release has been identified and named mediato-phore (see above). However, mediato-phore is a homo-oligomer of a 16 kDa proteolipid that is also part of the membrane sector of the V-ATPase. The 16-kDa element in itself cannot therefore be considered as a cholinergic-specific protein. What may be actually more specific for cholinergic neurons is the mechanism that directs the 16-kDa to the plasma membrane. This question was recently addressed by Leroy and Meunier [22] who expressed the 16-kDa in *Xenopus* oocytes. They showed that injecting the mRNA of the 16-kDa alone was less efficient in inducing ACh release than injecting the whole set of poly (A*) mRNAs extracted from cholinergic neurons. In the former, the 16-kDa protein accumulated

chiefly in intracellular membranes. With the total poly (A*) mRNAs, substantial amounts reached the plasmalemma. Therefore, another protein is probably required for directing the 16-kDa subunit at the plasma membrane where it is assembled to form the mediato-phore homo-oligomer. This process may coordinate the events associated with expression of the cholinergic locus and that of a specific step for ACh release. In this respect, cAMP-dependent systems may represent an important regulation pathway since they can upregulate both ACh synthesis and ACh release, as well as Ca^{2+} channels in some cells [3, 12, 20].

2.4. Vesicular docking mechanisms

The three proteins, syntaxin, SNAP-25 and VAMP/synaptobrevin constitute a clostridial toxin-sensitive site, since they are cleaved by botulinum and tetanus toxins. This complex has been purified by fractionation and immunoprecipitation experiments [29, 31]. There are, in addition, two possible systems that may anchor the vesicles to the plasma membrane: the 'C2' attachment site and the synaptophysin-mediato-phore link.

Several proteins share a homologous stretch of sequence, the C2 domain. It has been found in the vesicular protein synaptotagmin, in protein kinase C and in the cytoplasmic protein rabphilin. This last protein undergoes reversible binding to Rab3-GTP and thereby, to vesicle membranes. The C2 anchor binds directly or indirectly to the calcium channel as shown by immunoprecipitation experiments [17, 23]. Decoy peptides homologous to the C2 domain or protein kinase C itself perturb this C2 docking and alter transmission [5]. Similarly, interference with Rab-rabphilin attachment affects release in pituitary cells [24].

The third type of docking may involve two channel-forming proteins, synaptophysin and mediato-phore. This possibility was suggested by the fact that synaptophysin (and also synaptobrevin) can be immunoprecipitated with 16-kDa protein [15, 29] which is common to mediato-phore and to the c-subunit of the V-ATPase membrane sector (V0). In addition, another subunit of V-ATPase (39 kDa) was previously identified as physophilin, a synaptophysin-binding protein [30, 33]. This docking system might link in series, through mediato-phore and synaptophysin-V0, the vesicle lumen to the extracellular space. Such a link is expected to be resistant to clostridial toxins. It may still function, like the C2 anchor, when the clostridial anchor (T- and V-SNAREs) is absent or cut. In fact, vesicles still dock to the membrane when the clostridial link is damaged [32]. However, a physiologically intact

clostridial link is likely to be essential for controlling the shape and kinetics of the Ca^{2+} microdomains, needed for ensuring synchronization of release and protecting mediato-phores against desensitization. When this link is disrupted, quantal ACh release can still be elicited, but it becomes desynchronized and poorly Ca^{2+} -sensitive; hence, nerve-evoked transmission fails.

3. Conclusion

In order to develop its 'communication apparatus', a cholinergic neuron has to turn on several genes in parallel. The cholinergic locus provides ACh synthesis and storage. Mediato-phore molecules represent the elementary pores that translocate ACh either from the cytosol, or from vesicles if they are connected to the pore. The mediato-phores seem to be clustered at membrane spots [6], probable close to a calcium channel. This organization is not sufficient for an efficient and fully regulated transmission since the activity of individual mediato-phores has to be synchronized. The vesicles docked at the membrane by specific proteins in close contact to the calcium channel are important for this task. First, they efficiently take calcium, 'shaping' in space and time the local calcium microdomains which are determinant for phasic transmitter release. In addition, the vesicles perform a variety of controls according to the phase of their endo-exocytic cycle. They provide a reserve pool of transmitter. After a period of activity, they deliver, or take up, proteins and other compounds to, and from, the plasma membrane and the extracellular matrix.

References

- [1] Augustinsson K.B., Classification and comparative enzymology of the cholinesterases and methods for their determination, in: Koelle G.B. (Ed.), *Handbuch der experimentellen Pharmakologie*, Vol. XV, Springer Verlag, Berlin, 1963, pp. 89–128.
- [2] Berrard S., Varoqui H., Cervini R., Israël M., Mallet J., Diebler M.-F., Coregulation of two embedded gene products, choline acetyltransferase and the vesicular acetylcholine transporter, *J. Neurochem.* 65 (1995) 939–942.
- [3] Berse B., Blusztajn J.K., Coordinated up-regulation of choline acetyltransferase and vesicular acetylcholine transporter gene expression by the retinoic acid receptor α , cAMP, and leukemia inhibitory factor/ciliary neurotrophic factor signaling pathways in a murine septal cell line, *J. Biol. Chem.* 270 (1995) 22101–22104.
- [4] Béjanin S., Cervini R., Mallet J., Berrard S., A unique gene organization for two cholinergic markers, choline acetyltransferase and a putative vesicular transporter of acetylcholine, *J. Biol. Chem.* 269 (1994) 21944–21947.
- [5] Bommert K., Charlton M.P., DeBello W.M., Chin G.J., Betz H., Augustine G.J., Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis, *Nature* 363 (1993) 163–165.
- [6] Brochier G., Israël M., Lesbats B., Immunolabelling of the presynaptic membrane of Torpedo electric organ nerve terminals with an antiserum towards the acetylcholine releasing protein mediato-phore, *Biol. Cell* 78 (1993) 145–154.
- [7] Cavalli A., Dunant Y., Leroy C., Meunier F.M., Morel N., Israël M., Antisense probes against mediato-phore block transmitter release in oocytes primed with neuronal mRNAs, *Eur. J. Neurosci.* 5 (1993) 1539–1544.
- [8] Cavalli A., Eder-Colli L., Dunant Y., Loctin F., Morel N., Release of acetylcholine from *Xenopus* oocytes injected with nRNAs from cholinergic neurons, *EMBO J.* 10 (1991) 1671–1675.
- [9] Dan Y., Poo M.M., Quantal transmitter secretion from myocytes loaded with acetylcholine, *Nature* 359 (1992) 733–736.
- [10] Erickson J.D., Varoqui H., Schafer M.K., Modi W., Diebler M.F., Weihe E., Rand J., Eiden L.E., Bonner T.I., Usdin T.B., Functional identification of a vesicular acetylcholine transporter and its expression from a 'cholinergic' gene locus, *J. Biol. Chem.* 269 (1994) 21929–21932.
- [11] Falk-Vairant J., Corrèges P., Eder-Colli L., Salem N., Roulet E., Bloc A., Meunier F., Lesbats B., Loctin F., Synguelakis M., Israël M., Dunant Y., Quantal acetylcholine release induced by mediato-phore transfection, *Proc. Natl. Acad. Sci. USA* 93 (1996) 5203–5207.
- [12] Falk-Vairant J., Israël M., Bruner J., Stinnakre J., Meunier F.M., Gaultier P., Meunier F.A., Lesbats B., Synguelakis M., Corrèges P., Dunant Y., Evoked transmitter release from fibroblasts loaded with acetylcholine, enhancement by cAMP, *Neuroscience* 75 (1996) 353–360.
- [13] Falk-Vairant J., Meunier F.M., Lesbats B., Corrèges P., Eder-Colli L., Salem N., Synguelakis M., Dunant Y., Israël M., Cell lines expressing an acetylcholine release mechanism, correction of a release-deficient cell by mediato-phore transfection, *J. Neurosci. Res.* 45 (1996) 195–201.
- [14] Fisher L.J., Schinstine M., Salvaterra P., Dekker A.J., Thal L., Gage F.H., In vivo production and release of acetylcholine from primary fibroblasts genetically modified to express choline acetyltransferase, *J. Neurochem.* 61 (1993) 1323–1332.
- [15] Galli T., McPherson P.S., De Camilli P., The Vo sector of the V-ATPase, synaptobrevin, and synaptophysin are associated on synaptic vesicles in a Triton X-100-resistant, freeze-thawing sensitive, complex, *J. Biol. Chem.* 271 (1996) 2193–2198.
- [16] Girod R., Popov S., Alder J., Zheng J.Q., Lohof A., Poo M.M., Spontaneous quantal transmitter secretion from myocytes and fibroblasts: comparison with neuronal secretion, *J. Neurosci.* 15 (1995) 2826–2838.
- [17] Horikawa H.P., Saisu H., Ishizuka T., Sekine Y., Tsugita A., Odani S., Abe T., A complex of rab3A, SNAP-25, VAMP/synaptobrevin-2 and syntaxins in brain presynaptic terminals, *FEBS Lett.* 330 (1993) 236–240.
- [18] Israël M., Lesbats B., Morel N., Manaranche R., Gulik-Krzywicki T., Dedieu J., Reconstitution of a functional synaptic membrane possessing the protein constituents involved in acetylcholine translocation, *Proc. Natl. Acad. Sci. USA* 81 (1984) 277–281.
- [19] Israël M., Morel N., Lesbats B., Birman S., Manaranche R., Purification of a presynaptic membrane protein that mediates a calcium-dependent translocation of acetylcholine, *Proc. Natl. Acad. Sci. USA* 83 (1986) 9226–9230.

- [20] Israël M., Lesbats B., Synguelakis M., Joliot A., Acetylcholine accumulation and release by hybrid NG108-15, glioma and neuroblastoma cells - role of a 16 kDa membrane protein in release, *Neurochem. Int.* 25 (1994) 103-109.
- [21] Kimura Y., Oda Y., Deguchi T., Higashida H., Enhanced acetylcholine secretion in neuroblastoma x glioma hybrid NG108-15 cells transfected with rat acetyltransferase cDNA, *FEBS Lett.* 314 (1992) 409-412.
- [22] Leroy C., Meunier F.M., Differential targeting to the plasma membrane of the Torpedo 15-kDa proteolipid expressed in oocytes, *J. Neurochem.* 65 (1995) 1789-1797.
- [23] Leveque C., Hoshino T., David P., Shoji-Kasai Y., Leys K., Omori A., Lang B., El Far O., Sato K., Martin Moutot N., The synaptic vesicle protein synaptotagmin associates with calcium channels and is a putative Lambert-Eaton myasthenic syndrome antigen, *Proc. Natl. Acad. Sci. USA* 89 (1992) 3625-3629.
- [24] Lledo P.M., Vernier P., Vincent J.D., Mason W.T., Zorec R., Inhibition of Rab3B expression attenuates Ca^{2+} -dependent exocytosis in rat anterior pituitary cells, *Nature* 364 (1993) 540-544.
- [25] Miledi R., Reiser G., Uchitel O.D., Characteristics of membrane channels induced by acetylcholine at frog muscle-tendon junctions, *J. Physiol. (Lond.)* 350 (1994) 269-277.
- [26] Misawa H., Takahashi R., Deguchi T., Coordinate expression of vesicular acetylcholine transporter and choline acetyltransferase in sympathetic superior cervical neurones, *Neuroreport* 6 (1995) 965-968.
- [27] Morimoto T., Popov S., Buckley K.M., Poo M.M., Calcium-dependent transmitter secretion from fibroblasts: modulation by synaptotagmin I, *Neuron* 15 (1995) 689-696.
- [28] Rothman J.E., Mechanisms of intracellular protein transport, *Nature* 372 (1994) 55-63.
- [29] Shiff G., Synguelakis M., Morel N., Association of syntaxin with SNAP 25 and VAMP (synaptobrevin) in Torpedo synaptosomes, *Neurochem. Int.* 29 (1996) 659-667.
- [30] Siebert A., Lottspeich F., Nelson N., Betz H., Purification of the synaptic vesicle-binding protein physophilin; identification as the 39-kDa subunit of the vesicular H^{+} ATPase, *J. Biol. Chem.* 269 (1994) 28329-28334.
- [31] Sollner T., Rothman J.E., Neurotransmission: harnessing fusion machinery at the synapse, *Trends Neurosci.* 17 (1994) 344-348.
- [32] Sweeney S.T., Broadie K., Keane J., Niemann H., O'Kane C.J., Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects, *Neuron* 14 (1995) 341-351.
- [33] Thomas L., Betz H., Synaptophysin binds to physophilin, a putative synaptic plasma membrane protein, *J. Cell Biol.* 111 (1990) 2041-2052.
- [34] Whittaker V.P., Identification of acetylcholine and related esters of biological origin, in: Koelle G.B. (Ed.), *Handbuch der experimentellen Pharmacologie*, Vol XV, Springer Verlag, Berlin, 1963, pp. 1-39.
- [35] Birman S., Meunier F.M., Lesbats B., Le Cacr J.P., Rossier J., Israël M., A 15 kDa proteolipid found in mediatophore preparations from *Torpedo* electric organ presents high sequence homology with bovine chromaffin granule pronaphore, *FEBS Lett.* 261 (1990) 303-306.

Protein interactions implicated in neurotransmitter release

Oussama El Far^a, Vincent O'Connor^a, Thomas Dresbach^a, Lorenzo Pellegrini^a,
William DeBello^b, Felix Schweizer^b, George Augustine^b, Christian Heuss^c, Theo Schäfer^c,
Milton P. Charlton^d, Heinrich Betz^a

^a*Abteilung Neurochemie, Max-Planck-Institut für Hirnforschung, D-60528 Frankfurt, Germany*

^b*Department of Neurobiology, Duke University Medical Center, Durham, North Carolina 27710, USA*

^c*Friedrich-Miescher-Institut, CH-4002 Basel, Switzerland*

^d*Department of Physiology, University of Toronto, Ontario M5S 1A8, Canada*

Abstract — Biochemical evidence indicates that the exocytotic release of neurotransmitters involves both evolutionary conserved membrane proteins, the SNAREs, as well as ubiquitous cytosolic fusion proteins, NSF and SNAPs. We have analyzed the biochemical properties and the physiological effects of these proteins. Our data suggest models how NSF, SNAPs and SNAREs may function in neurotransmitter exocytosis. (©Elsevier, Paris)

Résumé — **Interactions protéiques impliquées dans la libération des neurotransmetteurs.** Des données biochimiques ont montré que des protéines membranaires (les SNAREs) et ubiquitaires cytosoliques (NSF et SNAPs) conservées au cours de l'évolution sont impliquées dans la libération des neurotransmetteurs. L'analyse des propriétés biochimiques et des effets physiologiques de ces protéines nous permettent de proposer des modèles sur la façon dont les SNAREs, les SNAPs et NSF pourraient être impliquées dans l'exocytose. (©Elsevier, Paris)

protein interactions / neurotransmitter release / SNAREs

1. Introduction

The regulated release of neurotransmitters at presynaptic nerve terminals provides the elementary process, upon which fast synaptic transmission is based. Its high speed and efficient regulation are crucial for intercellular communication on a millisecond time-scale. Work by Katz, Whittaker and others has established that this release process involves the Ca²⁺-regulated exocytosis of neurotransmitter from specialized storage organelles, the small synaptic vesicles. Rapid freezing of stimulated nerve terminals has allowed visualization of synaptic vesicles captured at various stages of fusion with the plasma membrane and identified a pool of synaptic vesicles that are clustered around specialized regions of the presynaptic plasmalemma [8]. These 'active zones' are thought to contain a sub-pool of readily releasable vesicles, which are 'docked' close to the channels that mediate the Ca²⁺ influx that triggers release [10, 11].

Recent biochemical and genetic studies indicate that an evolutionary conserved protein network utilized in all eukaryotic vesicle-based membrane traffic also mediates synaptic vesicle-plasma membrane fusion [3]. Experiments conducted to define the membrane receptors for ubiquitous cytosolic fusion factors, the ATPase *N*-ethylmaleimide sensitive fusion protein (NSF) and the α -, β - and γ -soluble NSF

attachment proteins (SNAPs), identified a synaptosomal complex composed of the plasma membrane proteins syntaxin and synaptosomal associated protein of 25 kDa (SNAP-25) and the synaptic vesicle protein synaptobrevin as the major SNAP binding component of rat brain extracts [23]. These proteins were therefore collectively named as SNAP receptors (SNAREs) and proposed to act as both synapse-specific membrane recognition molecules and acceptors for fusion catalyzing proteins.

In detergent extracts, the synaptosomal SNAREs form a complex that migrates at 7S upon density gradient centrifugation and is thought to represent the biochemical correlate of synaptic vesicles docked at the plasma membrane [23, 24]. Under conditions that prevent ATP hydrolysis, the SNARE complex binds SNAP and NSF to form a multimeric complex that migrates at 20S. ATP hydrolysis by NSF disrupts this 20S complex in a process that has been postulated to drive membrane fusion [23, 24]. Further evidence for an important role of the SNAREs in synaptic vesicle exocytosis comes from their identification as the targets of clostridial neurotoxins, the most potent inhibitors of neurotransmitter release known. The light chains of these toxins act as Zn²⁺ dependent endoproteases, with each light chain specifically cleaving one of the three synaptosomal SNAREs (*figure 1*). Synaptobrevin is cleaved by tetanus toxin and botulinum toxins B, D, F and G;

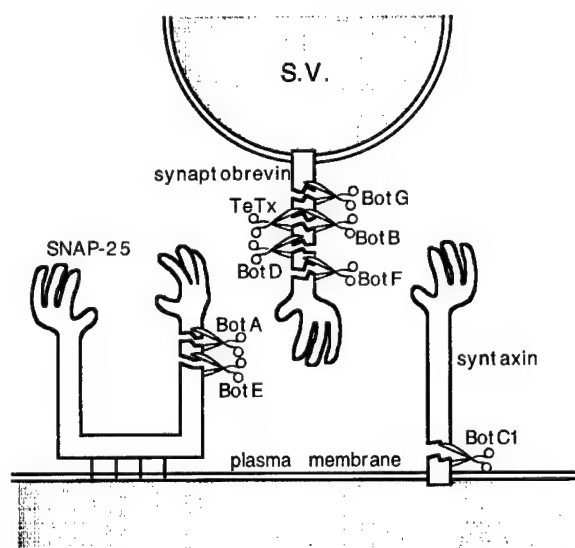


Figure 1. Clostridial toxin cleavage sites. The proteins cleaved by the clostridial neurotoxins are shown to highlight their juxtaposed membrane orientations in the synaptic vesicle (synaptobrevin) and plasma (syntaxin and SNAP-25) membranes. This oversimplifies the *in vivo* situation as fractions of cellular syntaxin and SNAP-25 are found on synaptic vesicles [25]. Transmembrane domains insert syntaxin and synaptobrevin into the membrane, whereas palmitoylation of cysteine residues is responsible for the membrane localization of SNAP-25. Note that Bot C1 may cleave both syntaxin and SNAP-25.

syntaxin by botulinum toxin C1; and SNAP-25 by botulinum toxins A and E [13, 15].

In this report we summarize biochemical and microinjection experiments aimed at demonstrating a physiological role of the SNAREs and the soluble fusion proteins in a living synapse and clarifying the importance of their various interactions.

2. SNAREs have a post-docking role in synaptic vesicle fusion

To elucidate whether the SNAREs synaptobrevin and syntaxin are involved in synaptic vesicle exocytosis, we used the giant synapse of the squid stellate ganglion. The enormous size of this synapse allows simultaneous recording of pre- and postsynaptic potentials in a preparation amenable to microinjection of recombinant proteins, peptides and other molecular probes. First, we tested the function of the synaptic vesicle SNARE synaptobrevin. cDNA cloning of squid synaptobrevin revealed that the cleavage site for the clostridial Zn²⁺ proteases tetanus toxin and botulinum toxin B light chain is

conserved in this invertebrate [9]. Upon microinjection into the giant terminal, both toxins produced a slow irreversible inhibition of neurotransmitter release that was prevented by coinjection of an inhibitor peptide that corresponds to the sequence of synaptobrevin that spans the cleavage site. Electron microscopy revealed that the toxin-poisoned terminals contained increased numbers of synaptic vesicles around their active zone profiles. Importantly, the number of vesicles apposed to the presynaptic membrane, i.e., the docked vesicle population, was significantly higher than in control injected terminals [9]. Thus, toxin cleavage severed synaptobrevin function at a late step of vesicle exocytosis, which lies downstream of the vesicle-plasma membrane recognition and/or docking process.

The same approach was also used to unravel a role for the plasma membrane SNARE syntaxin [17]. Presynaptic injection of the light chain of botulinum toxin C1 irreversibly blocked neurotransmission. This is consistent with the conservation of the botulinum toxin C1 cleavage site in the amino acid sequence of the squid protein deduced from cDNA. Moreover, injection of the highly conserved C-terminal H3 domain of syntaxin also produced a potent reversible inhibition of neurotransmitter release. Biochemical experiments revealed that the recombinant H3 domain competed syntaxin binding to SNAP-25 and thus prevented the formation of 20S fusion complexes. In contrast to observations made upon tetanus toxin light chain injection, however, ultrastructural analysis of nerve terminals microinjected with either botulinum toxin C1 or the H3 domain failed to reveal changes in the number or distribution of synaptic vesicles at presynaptic active zones. These data are consistent with syntaxin-SNAP-25 interactions functioning in an early reversible post-docking step of the exocytotic process [17].

In vitro data also support the conclusion that the SNAREs function at post-docking steps of exocytotic membrane fusion. SNAREs are accessible to proteolysis by clostridial neurotoxins only in their free non-complexed state [19]. Surprisingly, however, SNARE complex formation was not abolished in brain extracts, in which synaptobrevin was completely cleaved by tetanus toxin treatment [20]. Rather both cleavage fragments of synaptobrevin were recovered in the resultant 7S SNARE complex. Also, assembly and disassembly of the 20S complex, i.e., binding of SNAP and NSF by the complexed SNAREs and their dissociation upon NSF-catalyzed ATP hydrolysis, was indistinguishable in tetanus toxin cleaved and control preparations. Apparently, synaptobrevin cleavage does not disrupt the SNAREs' principal conformational transitions. However, a highly characteristic property of the complexed

SNAREs, their ability to form a SDS-resistant trimer, was severely impaired as revealed by a different temperature sensitivity to SDS dissociation [7, 20]. This shows that the stability, i.e., the low intrinsic energy, of the SNARE complex is altered upon incorporation of toxin-cleaved synaptobrevin. We have therefore proposed that clostridial neurotoxins impair SNARE function by compromising the ability of the SNARE complex to form an 'activated' high-energy intermediate upon NSF-catalyzed ATP hydrolysis state [20]. Accordingly, the conformational energy stored in this complex is postulated to drive membrane fusion upon Ca^{2+} triggering [16] of a release event. This idea is consistent with recent ultrastructural [6] and reconstitution [26] experiments, which assign NSF-driven ATP hydrolysis a central role in the generation of free SNAREs capable of forming parallel hairpin-like SNARE complexes postulated to be essential for close apposition of the membranes to be fused.

3. The soluble fusion proteins snap and nsf: determinants of the size and kinetics of neurotransmitter release?

We also have employed the squid giant synapse to test the functions of the ubiquitous soluble fusion proteins NSF and SNAP. The cloning of a full-length squid SNAP cDNA allowed to produce a recombinant squid SNAP protein [5]. Microinjection of either this squid SNAP or recombinant mammalian α -SNAP into the giant presynaptic terminal produced an enhancement of transmitter release as assayed by postsynaptic recording. This indicates that SNAP is a rate-limiting component of the release apparatus. In contrast, peptides corresponding to potentially surface-exposed regions of squid SNAP caused a rapid and reversible inhibition of synaptic vesicle exocytosis. Examination by imaging and electron microscopy of the effects of one of these peptides revealed that its blocking action was not related to alterations in Ca^{2+} influx, but correlated with an about 3-fold increase in the number of synaptic vesicles docked at the active zones of the presynaptic plasma membrane. Assuming that this accumulation of docked vesicles in the peptide-inhibited terminals was the consequence of a competitive inhibition of SNAP interaction with other components of the vesicle release machinery [1, 21], our data are consistent with a post-docking role of SNAP in synaptic vesicle exocytosis.

Cloning of sequences encoding the squid homologue of mammalian NSF [22] revealed that, like SNAREs and SNAPs, this trimeric ATPase is highly conserved in invertebrates, a conclusion that also e-

merged from studies in *Drosophila melanogaster*. There, the temperature-sensitive comatose mutant phenotype has been shown to result from point mutations in one of two NSF genes [18]. The protein sequence deduced from a partial squid NSF cDNA allowed to synthesize peptides corresponding to subdomains of the highly conserved D1 region which are predicted to be surface-exposed. Microinjection of two of these peptides into the presynaptic terminal of squid giant synapse produced not only a reduction in neurotransmitter release, but also slowed its kinetics [22]. Specifically, both peptides increased the synaptic delay and slowed both the onset and the decay of excitatory post-synaptic currents. These effects were highly specific, since substitution of a single glycine residue by glutamate in one of the peptides caused a loss of peptide inhibition and slowing. Interestingly, the same substitution is found in one of the aforementioned *Drosophila* comatose mutants [18]. These data indicate that NSF has a key role in the regulation of the release process. Biochemical experiments revealed that the active NSF peptides inhibited SNAP stimulation of NSF's ATPase activity [22]. Electron microscopy showed a modest increase in the number of synaptic vesicles directly docked at the presynaptic plasma membrane, whereas the more distant vesicle pool was severely depleted. This is consistent with NSF functioning not only in the release process, but also being required for replenishing the pool of vesicles associated with synaptic zones. In conclusion, our experiments suggest that NSF-catalyzed ATP hydrolysis regulates both the kinetics of the exocytotic process and other steps of intraterminal vesicle traffick.

4. Discussion

The work reported here shows that both the SNAREs and the soluble fusion proteins NSF and SNAP have essential roles in neurotransmitter release. From our electrophysiological and ultrastructural data, all these proteins may be concluded to act at post-docking steps of synaptic vesicle exocytosis (figure 2A). This is consistent with the original postulates of the SNARE hypothesis [23, 24], although Ca^{2+} triggering is likely to occur after SNAP/NSF action [4, 20]. Recent studies on constitutive membrane fusion in yeast, however, have challenged this view by showing that NSF action can precede SNARE complex formation [12, 14]. It therefore has been proposed that NSF acts as a chaperone [2] to unfold SNARE complexes after fusion and thus to generate reactivated SNAREs to drive another fusion cycle (figure 2B). This model is consistent with ultrastructural data on recombinant SNARE complexes [6]

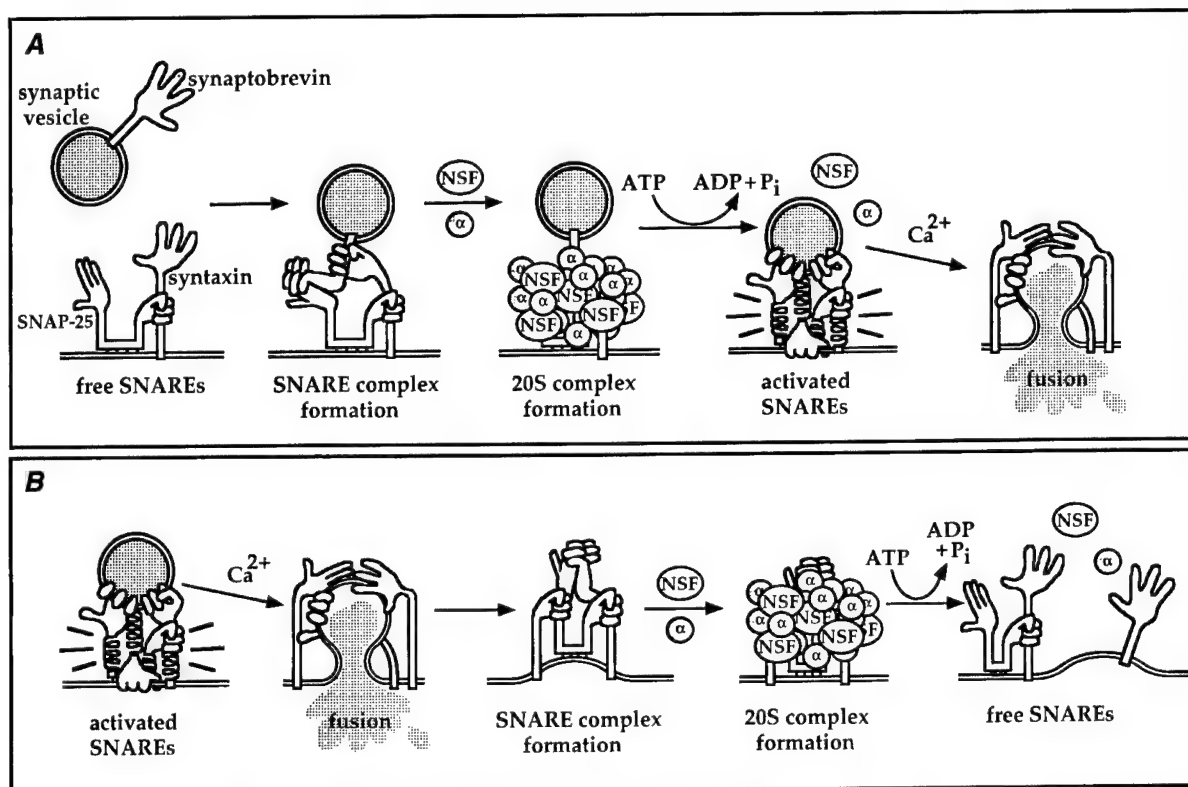


Figure 2. Pre- or post-fusion activation of SNARE complexes by NSF? **A.** Activation of SNARE complexes by NSF is assumed to occur after docking but prior to fusion. **B.** A post-fusion mechanism that reconciles data obtained from analysing SNARE complex function in yeast is shown. In both models, the activated complex is triggered by Ca^{2+} to initiate fusion. For **B**, this is postulated to cause a re-orientation of the SNAREs to result in a post-fusion complex that subsequently requires NSF action to liberate individual SNAREs. According to **B**, NSF generates a fusion-competent complex by re-activating SNAREs. The two models envisage NSF to act at distinct steps of the synaptic vesicle cycle. Model **B** is consistent with ultrastructural data obtained by negative staining [6].

which revealed a parallel orientation of synaptobrevin and syntaxin cytoplasmic domains. This parallel configuration might be crucial to overcome the energy barrier imposed by the repulsion of phospholipid head groups in the apposed lipid bilayers. Accordingly, the extraordinarily stable association of complexed SNAREs is a prerequisite for the fusion process [6, 26]. The effects of SNAP and NSF inhibition seen upon peptide injection into the squid giant synapse then might reflect a reduced number and/or interaction competence of partially reactivated SNAREs. A prediction of model **B** is that the 7S SNARE complex alone should be sufficient to drive membrane fusion. Recent reconstitution experiments with isolated SNAREs [26] are consistent with this interpretation.

Acknowledgments

We thank M. Baier and H. Reitz for secretarial assistance during the preparation of this manuscript, and Dr. D. Roth for critical reading. Work in the authors' laboratories was supported by Deutsche Forschungsgemeinschaft (Be and SFB 474), Fonds der Chemischen Industrie, Human Science Frontier Program Organisation, National Institutes of Health, Medical Research Council of Canada, and the Biomed and Health Research Program BMH1-CT93-1110.

References

- [1] Bommert K., Charlton M.P., DeBello W.M., Chin G.J., Betz H., Augustine G.J., Inhibition of transmitter release by C2

- domain peptides implicates synaptotagmin in exocytosis, *Nature* 363 (1993) 163–165.
- [2] Burgoyne RD., Morgan A., Ca^{2+} and secretory-vesicle dynamics, *Trends Neurosci.* 18 (1995) 191–196.
 - [3] Calakos N., Scheller H., Synaptic vesicle biogenesis, docking, and fusion: a molecular description, *Physiol. Rev.* 76 (1996) 1–29.
 - [4] DeBello W.M., Betz H., Augustine G.J., Synaptotagmin and neurotransmitter release, *Cell* 74 (1993) 947–50.
 - [5] DeBello W.M., O'Connor V., Dresbach T., Whiteheart S.W., Wang S.S.-H., Schweizer F.E., Betz H., Rothman J.E., Augustine G.J., SNAP-mediated protein-protein interactions essential for neurotransmitter release, *Nature* 373 (1995) 626–630.
 - [6] Hanson P.I., Roth R., Morisaki H., Jahn R., Heuser J.E., Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep etch electron microscopy, *Cell* 90 (1997) 523–535.
 - [7] Hayashi T., McMahon H., Yamasaki S., Binz T., Hata Y., Südhof T.C., Niemann H., Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly, *EMBO J.* 13 (1994) 5051–5061.
 - [8] Heuser J.E., Reese T.S., Dennis M.J., Jan Y., Jan L., Evans L., Synaptic vesicle exocytosis captured by quick-freezing and correlated with quantal transmitter release, *J. Cell Biol.* 81 (1979) 275–300.
 - [9] Hunt J.M., Bommert K., Charlton M.P., Kistner A., Habermann E., Augustine G.J., Betz H., A post-docking role for synaptobrevin in synaptic vesicle fusion, *Neuron* 12 (1994) 1269–1279.
 - [10] Léveque C., El Far O., Martin-Moutot N., Sato K., Kato R., Takahashi M., Seagar M.J., Purification of the N-type calcium channel associated with syntaxin and synaptotagmin, *J. Biol. Chem.* 262 (1994) 6306–6312.
 - [11] Llinas R., Sugimori M., Silver R.B., Microdomains of high calcium concentration in a presynaptic terminal, *Science* 256 (1992) 677–679.
 - [12] Mayer A., Wickner W., Haas A., Sec18p (NSF)-driven release of Sec17p (Alpha-SNAP) can precede docking and fusion of yeast vacuoles, *Cell* 89 (1996) 83–94.
 - [13] Montecucco C., Schiavo G., Structure and function of tetanus and botulinum neurotoxins, *Quart. Rev. Biophys.* 28 (1995) 423–472.
 - [14] Nichols B.J., Ungermann C., Peltham H.R., Wickner W.T., Haas A., Homotypic vacuolar fusion mediated by t- and v-SNAREs, *Nature* 387 (1997) 199–202.
 - [15] Niemann H., Blasi J., Jahn R., Clostridial neurotoxins: new tools for dissecting exocytosis, *Trends Cell. Biol.* 4 (1994) 179–185.
 - [16] O'Connor V., Augustine G.J., Betz H., Synaptic vesicle exocytosis: molecules and models, *Cell* 7 (1994) 785–787.
 - [17] O'Connor V., Heuss C., DeBello W.M., Dresbach T., Charlton M.P., Hunt J.H., Pellegrini L.L., Hodel A., Burger M.M., Betz H., Augustine G.J., Schäfer T., Disruption of syntaxin-mediated protein interactions blocks neurotransmitter secretion, *Proc. Natl. Acad. Sci. USA* 94 (1997) 12186–12191.
 - [18] Pallanck L., Ordway R.W., Ganetzky B., A *Drosophila* NSF mutant, *Nature* 376 (1995) 25.
 - [19] Pellegrini L.L., O'Connor V., Betz H., Fusion complex formation protects synaptobrevin against proteolysis by tetanus toxin light chain, *FEBS Lett.* 353 (1994) 319–323.
 - [20] Pellegrini L.L., O'Connor V., Lottspeich F., Betz H., Clostridial neurotoxins compromise the stability of a low energy SNARE complex mediating NSF activation of synaptic vesicle fusion, *EMBO J.* 14 (1995) 4705–4713.
 - [21] Schweizer F.E., Betz H., Augustine G.J., From vesicle docking to endocytosis: intermediate reaction of exocytosis, *Neuron* 14 (1995) 689–696.
 - [22] Schweizer F.E., Dresbach T., DeBello W.M., O'Connor V., Augustine G.J., Betz H., Regulation of neurotransmitter release kinetics by NSF, *Science* 279 (1998) 1203–1206.
 - [23] Söllner T., Bennet M.K., Whiteheart S.W., Scheller R.H., Rothman J.E. A., Protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation and fusion, *Cell* 75 (1993) 409–418.
 - [24] Söllner T., Whiteheart S.W., Brunner M., Erdjument-Bromage H., Geromanos S., Tempst P., Rothman J.E., SNAP receptors implicated in vesicle targeting and fusion, *Nature* 362 (1993) 318–324.
 - [25] Walch-Solimena C., Blasi J., Edelman L., Chapman E.R., von Mollard G.F., Jahn R., The t-SNAREs syntaxin 1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling, *J. Cell Biol.* 128 (1995) 637–645.
 - [26] Weber T., Zemelman B.V., Mc New I.A., Westermann B., Gmachl M., Parlati F., Söllner T.H., Rothman J.E., SNARE-pins: minimal machinery for membrane fusion, *Cell* 92 (1998) 759–772.

On the action of botulinum neurotoxins A and E at cholinergic terminals

Philip Washbourne^a, Rossella Pellizzari^a, Ornella Rossetto^a, Nicola Bortoletto^a,
Valeria Tugnoli^b, Domenico De Grandis^b, Roberto Eleopra^b, Cesare Montecucco^{a*}

^aCentro CNR Biomembrane and Dipartimento di Scienze Biomediche, Università di Padova, via G. Colombo 3, 35100 Padova, Italy

^bDivisione Neurologica, Arcispedale S. Anna, Corso Giovecca, Ferrara, Italy

Abstract — Botulinum neurotoxins type A and E (BoNT/A and /E) are metalloproteases with a unique specificity for SNAP-25 (synaptosomal-associated protein of 25 kDa), an essential protein component of the neuroexocytotic machinery. It was proposed that this specificity is based on the recognition of a nine-residue sequence, termed SNARE motif, which is common to the other two SNARE proteins: VAMP (vesicle-associated membrane protein) and syntaxin, the only known substrates of the other six clostridial neurotoxins. Here we report on recent studies which provide evidence for the involvement of the SNARE motif present in SNAP-25 in its interaction with BoNT/A and /E by following the kinetics of proteolysis of SNAP-25 mutants deleted of SNARE motifs. We show that a single copy of the motif is sufficient for BoNT/A and /E to recognise SNAP-25. While the copy of the motif proximal to the cleavage site is clearly involved in recognition, in its absence, other more distant copies of the motif are able to support proteolysis. We also report on studies of poisoning human neuromuscular junctions with either BoNT/A or BoNT/E and describe the unexpected finding that the time of recovery of function after poisoning is much shorter in the case of type E with respect to type A intoxication. These data are discussed in terms of the different sites of action of the two toxins within SNAP-25. (©Elsevier, Paris)

Résumé — Action des neurotoxines botuliniques A et E sur les terminaisons cholinergiques. Les neurotoxines botuliniques de type A et E (BoNT/A et BoNT/E) sont des métalloprotéases qui clivent de façon extrêmement spécifique la SNAP-25 ('synaptosomal associated protein of 25 kDa'), qui joue un rôle essentiel dans les processus de neuro-exocytose. Il a été proposé que cette spécificité repose sur la reconnaissance d'un motif de neuf acides aminés, appelé motif SNARE, qui se retrouve dans les deux autres protéines 'SNARE', la VAMP ('vesicle-associated membrane protein') et la syntaxine, car ces protéines constituent les seuls substrats connus des six neurotoxines clostridiales. Nous avons étudié la protéolyse de mutants de SNAP-25 dans lesquels le motif SNARE est délété, et nous montrons que ce motif est impliqué dans l'interaction de la protéine avec BoNT/A et BoNT/E. Une seule copie de ce motif est suffisante pour que ces neurotoxines reconnaissent la SNAP-25 : le motif le plus proche du site de clivage joue un rôle évident dans cette reconnaissance, mais en son absence, d'autres copies plus éloignées de ce motif peuvent assurer la protéolyse. Nous décrivons aussi l'effet neurotoxique de BoNT/A et BoNT/E sur des jonctions neuromusculaires humaines : de façon inattendue, la récupération fonctionnelle est beaucoup plus rapide après l'action de la BoNT/E qu'après intoxication par la BoNT/A. Nous discutons ces résultats en fonction des sites de clivage de la SNAP-25 par ces deux toxines. (©Elsevier, Paris)

SNAP-25 / SNARE motif / botulism / neurotoxin / zinc-endopeptidases / dystonia

1. Introduction

Botulinum neurotoxins cause the flaccid paralysis typical of botulism by blocking acetylcholine release at the neuromuscular junction. They are di-chain zinc endopeptidases secreted by different bacteria of the genus *Clostridium*. The neurotoxins reach their intracellular targets by translocating the light chain (50 kDa) into the cytosol after having been endocytosed via interaction of the heavy chain (100 kDa) with a high affinity receptor complex [7, 8]. Sero-

types B, D, F and G proteolyse VAMP/synaptobrevin [18, 20], serotypes A and E specifically cleave SNAP-25 [1, 2, 19] and type C acts on syntaxin and SNAP-25 [5, 11, 22]. Syntaxin and SNAP-25, associated with the cytoplasmic face of the plasma membrane of presynaptic nerve terminals, and VAMP, inserted into the synaptic vesicle membrane, have been postulated to act as synaptic vesicle docking receptors (t- and v-SNAREs). They have been implicated in the steps leading to membrane fusion for release of neurotransmitter into the synaptic cleft [16, 17]. SNAP-25, however, seems not only involved in neuroexocytosis, but is also required for axonal growth and synaptogenesis [10].

The three SNAREs, SNAP-25, syntaxin and VAMP are the only eukaryotic proteins to possess a distinct and conserved motif [14]. This short motif which is present at four positions in SNAP-25 (S1 to S4) (figure 1) is predicted to form an amphipathic

* Correspondence and reprints

Abbreviations: BoNT, botulinum neurotoxin; DTT, dithiothreitol; GST, glutathione S-methyl transferase; SNAP-25, synaptosome associated protein of 25 kDa; SNAREs, soluble NSF accessory protein receptors; TeNT, tetanus toxin; VAMP, vesicle associated membrane protein.

α -helix, providing a surface of negative charge on one-third of the surface and a group of hydrophobic residues on the adjacent third of the cylinder.

BoNT/A is the best available therapeutic agent of a variety of human diseases that benefit from a functional inhibition of cholinergic terminals [6, 9]. Hence, there is a particular importance in understanding the molecular basis of the action of BoNT/A. Here, we report on mutagenesis studies of SNAP-25 which demonstrate that recognition of the SNARE motif is essential for SNAP-25 proteolysis by BoNT/A and /E.

2. Materials and methods

BoNT/A and /E were obtained from Wako (Germany) and further purified via immobilized-metal-ion affinity chromatography to remove traces of contaminant proteases [15]. Anti C-terminal SNAP-25 antibodies were prepared as detailed previously [13]. Primary antibodies were detected by immunostaining with an anti-rabbit conjugated with alkaline phosphatase (Sigma) using nitroterrazolium blue and bromo-chloro-indolylphosphate (Sigma). Bacterial strains, plasmid construction and SNAP-25 deletions and mutagenesis were as described before [21].

2.1 Proteolytic assays

Mouse SNAP-25 and its mutants were expressed as GST fusion proteins and were purified by affinity chromatography on GSH-sepharose matrix (Pharmacia) as before [12]. After treatment with 10 mM DTT for 30 min at 37 °C, BoNT/A or BoNT/E (50 nM final concentration) were added to each of the GST-fusion SNAP-25s (0.5 μ g/ μ L final concentration) in phosphate buffer: 10 mM NaH_2PO_4 , 150 mM NaCl, pH 7.4 and incubated at 37 °C for 60 min. The concentrations of the two proteases were selected after preliminary experiments to provide rates of cleavage capable of revealing differences among SNAP-25 mutants. Samples were analysed by Western blotting onto nitrocellulose after electrophoresis in a 13% polyacrylamide SDS gel and were probed with an anti SNAP-25 C-terminal antibody.

2.2 Neuromuscular block with BoNT/A and /E

The EDB muscles of human volunteers were injected with BoNT/A in one side and with BoNT/E in the other side as described before [3]. At 7, 14, 30, 45, 60 and 90 days the recovery of neuromuscular function was assayed by measuring muscular contraction elicited by a supramaximal stimulation of the peroneal muscle.

3. Results and discussion

Figure 1 illustrates the location and sequence of the four copies of the SNARE motif present in the SNAP-25 molecule, together with the cleavage sites

of BoNT/A and /E. Beginning from the amino-terminus, the four copies of the motif are termed S1, S2, S3 and S4. To determine which of the four copies of the SNARE motif present in neuronal SNAP-25 is important for the recognition by the SNAP-25 specific clostridial neurotoxins, we constructed a series of N-terminal deletions of mouse SNAP-25b expressed as recombinant GST-fusion proteins and measured their rate of proteolytic cleavage. Figure 1 also shows the various chimeric GST-SNAP-25 constructs that have been prepared (top panel), expressed in *E. coli* and purified by affinity chromatography on Sepharose-GSH columns, together with the extent of their proteolytic cleavage by BoNT/A (left panel) and BoNT/E (right panel) after 1 h of incubation. Progressive deletions beginning from the distal amino-terminal copy of the motif do not alter the rate of cleavage of the substrate both by BoNT/A and by BoNT/E. Moreover, the deleted SNAP-25 substrates are cleaved as long as a single copy of the SNARE motif (S4) is present. However, removal of a further 14 residues containing the S4 motif from $\Delta 1$ –140 to produce $\Delta 1$ –154 totally abolishes proteolytic activity. Hence, the minimal segment of the SNAP-25 molecule required for the proteolytic cleavage by both BoNT/A and /E has to include S4.

To determine whether the other SNARE motif copies present in the SNAP-25 molecule could substitute for S4, N-terminal deletions were generated using a Δ S4 mutant (figure 2). As shown, the rate of cleavage by BoNT/A is reduced progressively for each SNARE motif removed, but cleavage is completely abolished when all remaining SNARE motifs were deleted. BoNT/E shows a smaller dependence of the rate of proteolysis on sequential removal of the N-terminal SNARE motifs, but it is entirely inactive on a SNAP-25 mutant not containing any copy of the motif. These results indicate that the N-terminal SNARE motifs, S1, S2 and S3, can substitute to different extents for the absence of S4.

BoNT/A is the best available treatment for a variety of human dystonias and other cholinergic syndromes [9]. However, a sizeable proportion of patients are resistant to BoNT/A from the first injection, while others become resistant following the production of antibodies specific for BoNT/A. For this reason, we have introduced the use of BoNT/C and have proven that this toxin is as effective as BoNT/A with therapeutic benefits lasting for just as long [4]. To extend this approach, we have tested the effect of BoNT/E on humans. This toxin induces a very effective paralysis in humans. However, neuromuscular junctions of EDB muscles recovered their full function after 30–45 days after the injection of BoNT/E, compared to the more than 90 days needed to recover from BoNT/A treatment. This result

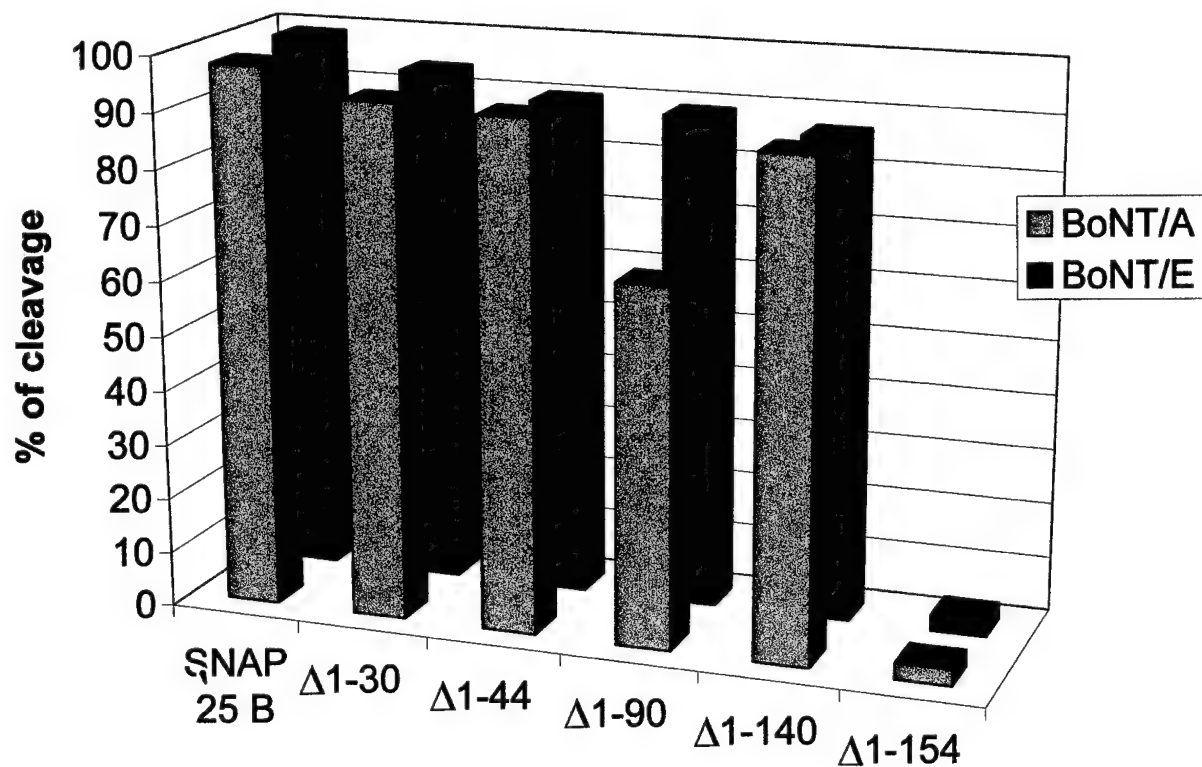
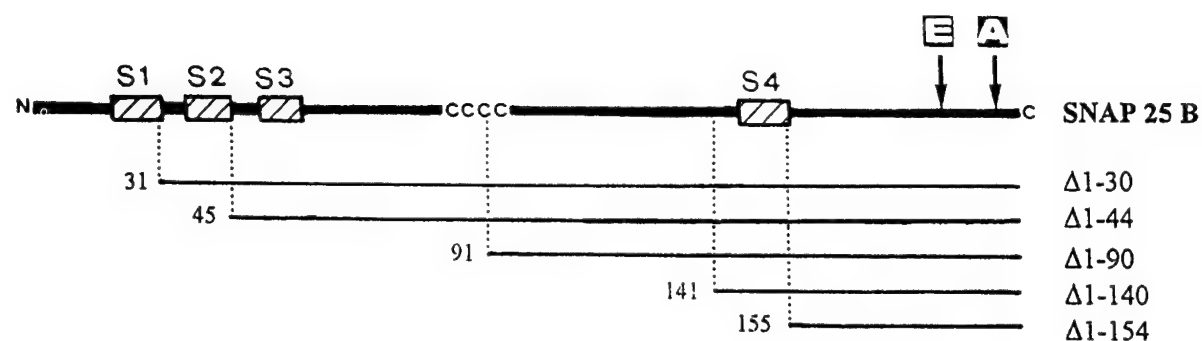


Figure 1. Proteolysis by BoNT/A and /E of N-terminal deletion mutants of SNAP-25. The upper diagram shows the deletion mutants that have been fused to GST and assayed for their susceptibility to BoNT/A and /E. All constructs were incubated with 50 nM BoNT/A and 50 nM BoNT/E, samples were taken after 60 min, submitted to electrophoresis and Western blot. The alkaline phosphatase stained blots were quantified by densitometric scanning. Data are average of three independent experiments.

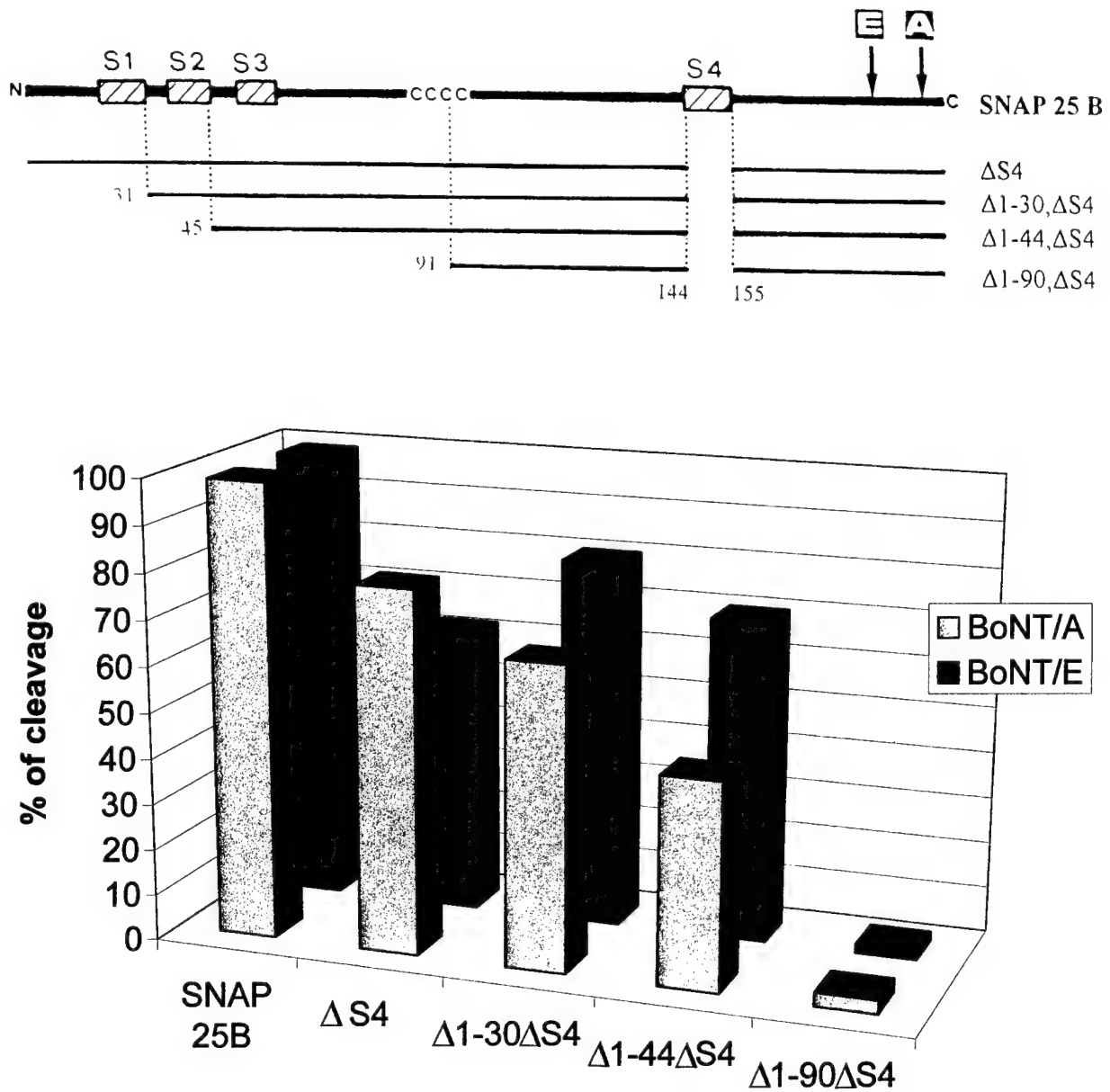


Figure 2. Neurotoxin proteolysis of deletion mutants that progressively remove the three N-terminal SNARE motifs in the absence of S4. The upper diagram shows deletion mutants of SNAP-25, but which include a loop out deletion of amino acids 141 to 154 (Δ S4). All constructs were incubated with 50 nM BoNT/A and 50 nM BoNT/E, samples were taken after 60 min, submitted to electrophoresis and Western blot. The alkaline phosphatase stained blots were quantified by densitometric scanning. Data are average of three independent experiments.

is not due to a different life-time of the two toxins inside the motoneuron terminals. Rather, it appears that removal of 26 residues, caused by BoNT/E, from the SNAP-25 C-terminus induces a much more rapid replacement of the damaged SNAP-25 molecule with respect to the removal of nine residues, caused by BoNT/A.

Acknowledgments

This study was supported by Telethon Italia grant 763 and by MURST 60%.

References

- [1] Binz T., Blasi J., Yamasaki S., Baumeister A., Link E., Südhof T.C., Jahn R., Niemann H., *J. Biol. Chem.* 269 (1994) 1617–1620.
- [2] Blasi J., Chapman E.R., Link E., Binz T., Yamasaki S., De Camilli P., Südhof T.C., Niemann H., Jahn R., *Nature* 365 (1993) 160–163.
- [3] De Grandis D., Montecucco C., Rossetto O., Tugnoli V., Eleopra R., *Neurology* 50 (1998) A306.
- [4] Eleopra R., Tugnoli V., Rossetto O., Montecucco C., De Grandis D., *Neurosci. Lett.* 224 (1997) 91–94.
- [5] Foran P., Lawrence G.W., Shone C.C., Foster K.A., Dolly J.O., *Biochemistry* 35 (1996) 2630–2636.
- [6] Jankovic J., Hallett M., *Therapy with Botulinum Toxin*, Marcel Dekker Inc, 1994.
- [7] Montecucco C., Schiavo G., *Mol. Microbiol.* 13 (1994) 1–8.
- [8] Montecucco C., Schiavo G., *Q. Rev. Biophys.* 28 (1995) 423–472.
- [9] Montecucco C., Schiavo G., Tugnoli V., Degrandis D., *Mol. Med. Today* 2 (1996) 418–424.
- [10] Osen Sand A., Catsicas M., Staple J.K., Jones K.A., Ayala G., Knowles J., Grenningloh G., Catsicas S., *Nature* 364 (1993) 445–448.
- [11] Osen Sand A., Staple J.K., Naldi E., Schiavo G., Rossetto O., Petitpierre S., Malgaroli A., Montecucco C., Catsicas S., *J. Comp. Neurol.* 367 (1996) 222–234.
- [12] Pellizzari R., Rossetto O., Lozzi L., Giovedi S., Johnson E., Shone C.C., Montecucco C., *J. Biol. Chem.* 271 (1996) 20353–20358.
- [13] Rossetto O., Gorza L., Schiavo G., Schiavo N., Scheller R.H., Montecucco C., *J. Cell. Biol.* 132 (1996) 167–179.
- [14] Rossetto O., Schiavo G., Montecucco C., Poulain B., Deloye F., Lozzi L., Shone C.C., *Nature* 372 (1994) 415–416.
- [15] Rossetto O., Schiavo G., Polverino de Laureto P., Fabbiani S., Montecucco C., *Biochem. J.* 285 (1992) 9–12.
- [16] Rothman J.E., Warren G., *Curr. Biol.* 4 (1994) 220–233.
- [17] Rothman J.E., Wieland F.T., *Science* 272 (1996) 227–234.
- [18] Schiavo G., Malizio C., Trimble W.S., Polverino de Laureto P., Milan G., Sugiyama H., Johnson E.A., Montecucco C., *J. Biol. Chem.* 269 (1994) 20213–20216.
- [19] Schiavo G., Santucci A., Dasgupta B.R., Mehta P.P., Jontes J., Benfenati F., Wilson M.C., Montecucco C., *FEBS Lett.* 335 (1993) 99–103.
- [20] Schiavo G., Shone C.C., Rossetto O., Alexander F.C., Montecucco C., *J. Biol. Chem.* 268 (1993) 11516–11519.
- [21] Washbourne P., Pellizzari R., Baldini G., Wilson M.C., Montecucco C., *FEBS Lett.* 418 (1997) 1–5.
- [22] Williamson L.C., Halpern J.L., Montecucco C., Brown J.E., Neale E.A., *J. Biol. Chem.* 271 (1996) 7694–7699.

Dissociation of the vesicular acetylcholine transporter domains important for high-affinity transport recognition, binding of vesamicol and targeting to synaptic vesicles

H. Varoqui, J.D. Erickson

*Neuroscience Center and Department of Pharmacology, Louisiana State University Medical School, 2020 Gravier Street, Suite D,
New Orleans, Louisiana 70112, USA*

Abstract — Chimeras between the human vesicular acetylcholine transporter (hVACHT) and the neuronal isoform of the human vesicular monoamine transporter (hVMAT2) have been constructed and stably expressed in a rat pheochromocytoma cell line (PC12) in an effort to identify cholinergic-specific domains of VACHT. Examination of the transport properties of a chimera in which the N-terminal portion (up to putative transmembrane domain II and including the lumenal glycosylated loop) of hVACHT was replaced with hVMAT2 sequences (2/V@NheI) revealed that its apparent affinity for acetylcholine (ACh) was reduced approximately seven-fold compared to wild-type. However, the affinity of this chimera for vesamicol did not significantly differ from hVACHT. Similarly, the 2/V@NheI chimera retained its preferential targeting to the small synaptic-like vesicles found in PC12 cells in agreement with our recently reported observations that the synaptic vesicle targeting domain resides in the cytoplasmic tail of VACHT. (©Elsevier, Paris)

Résumé — Dissociation des domaines du transporteur vésiculaire d'acétylcholine impliqués dans la reconnaissance du substrat, la liaison du vesamicol, et l'adressage vers les vésicules synaptiques. Nous avons cherché à identifier les régions spécifiquement cholinergiques du transporteur vésiculaire d'acétylcholine (VACHT) à l'aide de protéines chimériques humaines composées de fragments de hVACHT et de l'isoforme neuronale du transporteur vésiculaire de monoamines (hVMAT2), exprimées de façon stable dans des cellules PC12 (pheochromocytomes de rat). L'affinité apparente pour l'acétylcholine de la chimère 2/V@NheI (hVMAT2 jusqu'au début du domaine transmembranaire II, puis hVACHT) est réduite 7 fois par rapport à hVACHT. L'affinité de cette chimère pour le vesamicol est quant à elle inchangée. De plus, cette chimère 2/V@NheI, comme hVACHT, est adressée préférentiellement vers les petites vésicules de type synaptiques dans les cellules PC12. Ce résultat est en accord avec notre récente observation que le signal d'adressage vers les vésicules synaptiques se situe à l'extrémité C-terminale cytoplasmique de hVACHT. (©Elsevier, Paris)

vesicular acetylcholine transporter (VACHT) / neuronal isoform of the vesicular monoamine transporter (VMAT2) / acetylcholine / vesamicol / synaptic vesicles / targeting

1. Introduction

The vesicular acetylcholine transporter (VACHT) is responsible for packaging cytoplasmic acetylcholine (ACh) into the small synaptic vesicles of cholinergic neurons where it is available for regulated neurosecretion. Vesamicol, a neuromuscular blocking agent, disrupts this process by specifically inhibiting vesicular accumulation of ACh. Recently, the molecular cloning and functional identification of VACHT has revealed that vesicular ACh transport and vesamicol binding properties are encoded by a single polypeptide [11].

VACHT exhibits approximately 40% identity with the two other members of this gene family, the endocrine-specific (VMAT1) and neuronal (VMAT2) isoforms of the vesicular monoamine transporter [8]. Since these proteins all utilize a common H⁺/antiport mechanism for vesicular substrate accumulation, chimeric transporters offer an opportunity to identify regions of these proteins which are important in de-

termining substrate and inhibitor specificity as well as specific targeting to cholinergic versus monoaminergic secretory organelles.

Rat pheochromocytoma cells (PC12) synthesize both dopamine and ACh with expression of rVMAT1 on large dense core vesicles (LDCV) and rVACHT on the small synaptic vesicle clusters which can be observed following treatment with nerve growth factor [13]. Recently, we have shown that structural information resides within the terminal cytoplasmic domain of hVACHT, which specifically targets it to the PC12 synaptic vesicles [12]. Overexpression of hVACHT in PC12 cells results in approximately a 20-fold increase in vesamicol-sensitive vesicular accumulation of ACh [10]. This expression system should prove useful for the structure/function analysis of VACHT and help to further establish a relationship between the activity of vesicular neurotransmitter transporters and vesicular storage and levels of neurotransmitter available for regulated neurosecretion.

2. Materials and methods

Polyclonal antipeptide antibodies against the C-terminal amino acids of hVACHT [6] were affinity purified and previously characterized [10, 12]. Polyclonal antibodies against chromogranin B (CgB) were a generous gift of Reiner Fischer-Colbrie (Innsbrück, Austria). Monoclonal antibodies directed against synaptophysin (p38) were purchased from Sigma.

2.1. 2/V@NheI chimeric construction

An *NheI* restriction site was introduced into both hVACHT and hVMAT2 cDNAs at an equivalent site within the putative 2nd transmembrane domain of these proteins (conserved amino acids Ala¹⁴⁹ and Ser¹⁵⁰) by site-directed mutagenesis [4]. Mutagenesis was performed using the full-length hVMAT2 cDNA in pCDM7amp. Since hVACHT has two internal *NheI* sites, the 5' *HindIII/Sse8387I* fragment of hVACHT was first subcloned into pUC18 where mutagenesis was performed. Transformants were screened by restriction analysis and verified by cDNA double-stranded sequencing using Sequenase II (U.S. Biochemical Corp.). The 5' region of hVMAT2 was then subcloned into the hVACHT fragment (at *HindIII/NheI* in pUC18) and the 2/V chimeric fragment (*HindIII/Sse8387I*) was subcloned back into hVACHT in the mammalian expression vector Rc/CMV (Invitrogen).

2.2. Transfection and selection of stable PC12 cell lines

Rat PC12 cells were transfected with the 2/V@NheI construct using Lipofectin (10 mg/mL: Life Technologies, Inc.), and stable transformants were selected with 0.5 mg/mL Geneticin (G418; Life Technologies, Inc.) and screened by immunocytochemistry as previously described [10, 12].

2.3. Vesicular acetylcholine transport and vesamicol binding assays

ATP-dependent vesicular transport of [³H]-ACh (55.2 mCi/mmol, DuPont NEN) and the binding of [³H]-vesamicol (AH5183, L-[piperidinyl-3,4-³H]vesamicol; 31 Ci/mmol, DuPont NEN) were measured using postnuclear supernatants (800 g for 10 min) prepared from control, hVACHT-expressing or 2/V@NheI-expressing PC12 cells as previously described [9, 10]. Uptake of ³H-acetylcholine by control PC12 cells was subtracted. Non-specific binding of ³H-vesamicol was determined in the presence of 30 μ M L-vesamicol and was subtracted from the total binding.

2.4. Subcellular localization

Postnuclear supernatants were centrifuged at 10 000 g for 10 min, and the resulting supernatant (about 2 mg of protein) was loaded onto a 4.6 mL 5–25% glycerol gradient prepared in homogenization buffer for a 45-min-long centrifugation at 55 000 rpm (SW55 rotor), which enables separation of endosomal membranes from synaptic vesicles [2]. Successive 350- μ L fractions were collected and processed for Western blot analysis as previously described [12].

3. Results

The hVACHT and 2/V@NheI chimera could be distinguished from the endogenously expressed rVACHT in stably transfected PC12 cells lines by immunocytochemistry and Western blotting using a human-specific antibody. Furthermore, the binding of [³H]vesamicol and transport of [³H]ACh in postnuclear supernatants of PC12 cells expressing the human proteins was significantly higher than levels detected in PC12 cells containing only the endogenous rVACHT. Uptake mediated by the endogenous rVACHT protein was less than two-fold greater than that observed in the presence of vesamicol or at 4 °C.

3.1. ACh transport and vesamicol binding properties of 2/V@NheI chimera

Specific vesicular uptake of [³H]ACh was observed in homogenates from the 2/V@NheI chimera. Uptake of [³H]ACh was completely inhibited by 2 mM L-vesamicol, bafilomycin A₁ (1 μ M), a specific inhibitor of the vesicular H⁺ ATPase, and the proton ionophore FCCP (2.5 μ M). Furthermore, [³H]ACh transport was reduced approximately 90% in the absence of exogenous ATP.

The kinetic analysis of the uptake of [³H]ACh by the 2/V@NheI chimera is shown in figure 1A. The initial rate of [³H]ACh uptake was measured during the linear portion of the time course (6 min) and was saturable and displayed an apparent *K_m* of 7.4 mM. The apparent affinity of hVACHT for ACh (*K_m* = 0.9 mM) is shown for comparison. Thus, the presence of hVMAT2 sequences in the N-terminal portion of hVACHT significantly reduces the affinity of this chimeric transporter for ACh. The 2/V@NheI chimera does not however transport monoamines when expressed in digitonin-permeabilized CV-1 fibroblasts (data not shown).

The affinity of [³H]vesamicol for the 2/V@NheI chimera was not significantly different from that observed with hVACHT and exhibited a *K_d* of approximately 5 nM (figure 1B).

3.2. Subcellular localization of 2/V@NheI chimera

Western blot analysis of fractions obtained following velocity sedimentation of high speed supernatants of stably transfected PC12 cells through glycerol is shown in figure 2. The 2/V@NheI immunoreactivity sedimented in synaptic vesicles fractions containing synaptophysin (p38), similarly to that observed with hVACHT-expressing PC12 homogenates. Thus, the presence of hVMAT2 sequences in the N-terminal portion of hVACHT does not in-

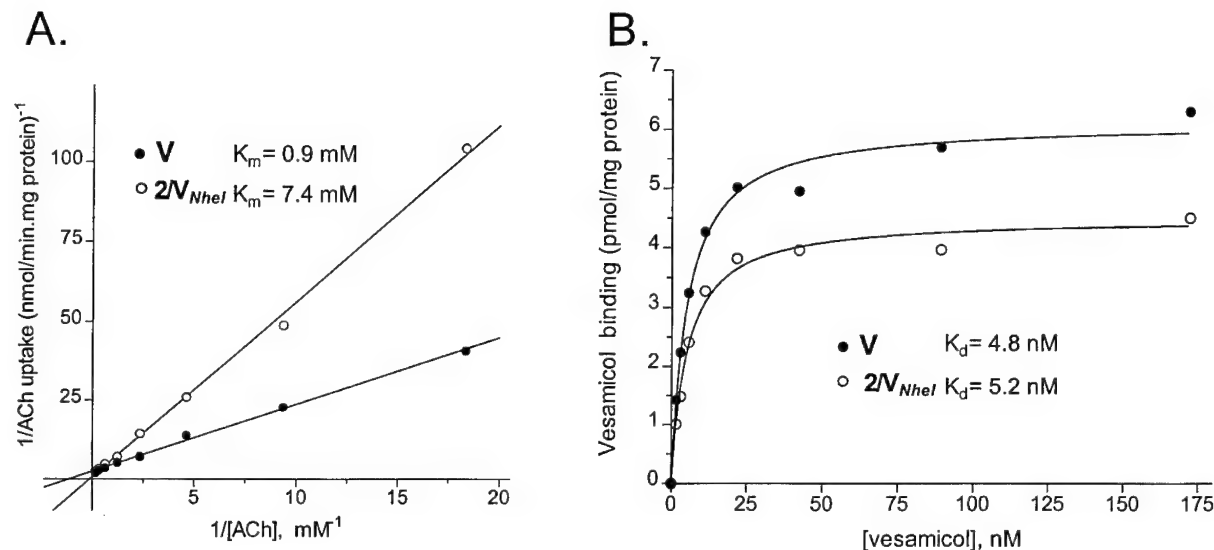


Figure 1. 2V@NheI chimera dissociates high-affinity ACh recognition from vesamicol binding. **A.** Lineweaver-Burk analysis of initial $[^3\text{H}]\text{ACh}$ (0.03–10 mM) uptake velocity. **B.** Saturation isotherm of binding of $[^3\text{H}]\text{vesamicol}$ (0.08–116 nM). Data are from a representative experiment performed in duplicate.

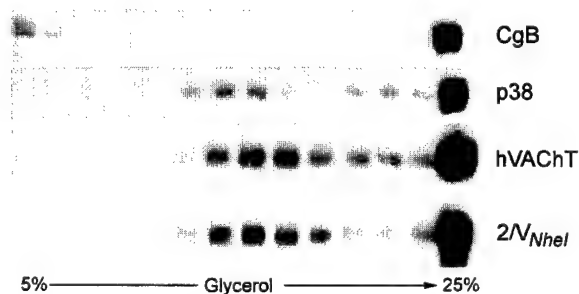


Figure 2. 2V@NheI chimera retains targeting to PC12 synaptic vesicles. Membrane protein from synaptic vesicle enriched supernatants fractionated through glycerol were immunoblotted with affinity-purified anti-hVAcHT. Each gradient was controlled for p38 and CgB localization.

terfer with its targeting to the small synaptic vesicles in PC12 cells.

4. Discussion

The results presented here indicate that high-affinity vesicular transport of ACh requires cholinergic-specific amino acids within the N-terminal portion of hVAcHT. It is likely that this specificity lies within the first putative transmembrane domain

(TMD) as the amino terminus and large luminal glycosylated loop between TMDs I and II are poorly conserved. The conserved aspartic acid residue (Asp³³) in TMD I of VMATs has been implicated in high-affinity monoamine recognition [5]. Our data suggest that other amino acids within TMD I of VAcHT, in addition to the aspartic acid residue (Asp⁴⁶), are also important for ACh transport function.

The binding of vesamicol does not require these cholinergic-specific amino acids in the N-terminal portion of hVAcHT. Vesamicol is a non-competitive inhibitor of active ACh transport in synaptic vesicles isolated from *Torpedo* [1, 3]. Our results are consistent with earlier work suggesting that ACh and vesamicol do not bind to the same site in hVAcHT. However, preliminary evidence suggests the presence of a second, lower affinity ACh binding site in hVAcHT which may interact with vesamicol.

It has recently been observed that the VAcHT-specific aspartic acid residue (Asp¹⁹³) in TMD IV and the conserved aspartic acid residue (Asp³⁹⁸) in TMD X are required for ACh transport function [7]. We are currently examining the role these residues play in high- and low-affinity ACh recognition and in the binding of vesamicol. The intramembrane aspartic acid residues as well as other polar amino acids (e.g., serine, glycine, cysteine) of VAcHT are likely to be part of a 'pore' and be important for the binding and exchange of H⁺ and ACh.

When expressed in PC12 cells, hVAcHT is preferentially targeted to the small synaptic vesicles while hVMAT2 is confined to the LDCVs [12]. Here we show that the presence of the luminal glycosylated loop of hVMAT2 does not interfere with the targeting of the 2/V@NheI chimera to the small synaptic vesicles. The synaptic vesicle targeting information instead resides within the terminal cytoplasmic domain of VAcHT [12]. Future work will determine what consequence a reduced affinity of hVAcHT for ACh has on regulated ACh secretion from the small synaptic vesicles of PC12 cells.

Acknowledgment

This work was supported by a grant from the National Institutes of Health (NS36936).

References

- [1] Bahr B.A., Parsons S.M., Acetylcholine transport and drug inhibition kinetics in *Torpedo* synaptic vesicles, *J. Neurochem.* 46 (1986) 1214–1218.
- [2] Clift-O'Grady L., Linstedt A.D., Lowe A.W., Grote E., Kelly R.B., Biogenesis of synaptic vesicle-like structures in a pheochromocytoma cell line PC-12, *J. Cell Biol.* 110 (1990) 1693–1703.
- [3] Diebler M.F., Morot Gaudry-Talarmin Y., AH5183 and cetylpyridinium: two potent inhibitors of acetylcholine uptake into synaptic vesicles from *Torpedo marmorata*, *J. Neurochem.* 52 (1989) 813–821.
- [4] Deng W.P., Nickoloff J.A., Site-directed mutagenesis of virtually any plasmid by eliminating a unique site, *Anal. Biochem.* 200 (1992) 81–85.
- [5] Merickel A., Rosandich P., Peter D., Edwards R.H., Identification of residues involved in substrate recognition by a vesicular monoamine transporter, *J. Biol. Chem.* 270 (1995) 25798–25804.
- [6] Schäfer M.K.-H., Weihe E., Erickson J.D., Eiden L.E., Human and monkey cholinergic neurons visualized in paraffin-embedded tissues by immunoreactivity for VAcHT, the vesicular acetylcholine transporter, *J. Mol. Neurosci.* 6 (1995) 225–235.
- [7] Song H.-J., Ming G.-L., Fon E., Bellocchio E., Edwards R.H., Poo M.-M., Expression of a putative vesicular acetylcholine transporter facilitates quantal transmitter packaging, *Neuron* 18 (1997) 815–826.
- [8] Usdin T.B., Eiden L.E., Bonner T.I., Erickson J.D., Molecular biology of the vesicular ACh transporter, *Trends Neurosci.* 18 (1995) 218–224.
- [9] Varoqui H., Diebler M.-F., Meunier F.-M., Rand J.B., Usdin T.B., Bonner T.I., Eiden L.E., Erickson J.D., Cloning and expression of the vesamicol binding protein from the marine ray *Torpedo*: homology with the putative vesicular acetylcholine transporter UNC-17 from *Caenorhabditis elegans*, *FEBS Lett.* 342 (1994) 97–102.
- [10] Varoqui H., Erickson J.D., Active transport of acetylcholine by the human vesicular acetylcholine transporter, *J. Biol. Chem.* 271 (1996) 27229–27232.
- [11] Varoqui H., Erickson J.D., Vesicular neurotransmitter transporters: potential sites for the regulation of synaptic function, *Mol. Neurobiol.* 15 (1997) 165–191.
- [12] Varoqui H., Erickson J.D., The cytoplasmic tail of the vesicular acetylcholine transporter contains a synaptic vesicle targeting signal, *J. Biol. Chem.* 273 (1998) 9094–9098.
- [13] Weihe E., Tao-Cheng J.-H., Schäfer M.K.-H., Erickson J.D., Eiden L.E., Visualization of the vesicular acetylcholine transporter in cholinergic nerve terminals and its targeting to a specific population of small synaptic vesicles, *Proc. Natl. Acad. Sci. USA* 93 (1996) 3547–3552.

The cholinergic locus: ChAT and VAcHT genes

Jacques Mallet, Leïla Houhou, Fabrice Pajak, Yoshio Oda*, Ricardo Cervini**,
Stéphane Bejanin***, Sylvie Berrard

CNRS, Laboratoire de Génétique de la Neurotransmission et des Processus Neurodégénératifs,
Hôpital Pitié-Salpêtrière, 75013 Paris, France

Abstract — The gene encoding the vesicular acetylcholine transporter has been localized within the first intron of the gene encoding choline acetyltransferase and is in the same transcriptional orientation. These two genes, whose products are required for the expression of the cholinergic phenotype, could therefore be coregulated. The promoters of both genes have been identified. The mechanisms that account for the regulation of the expression of both genes are now being investigated. (©Elsevier, Paris)

Résumé — Le locus cholinergique: les gènes ChAT et VAcHT. Le gène codant le transporteur vésiculaire de l'acétylcholine a été localisé dans le premier intron du gène codant la choline acétyltransférase, dans la même direction transcriptionnelle que celui-ci. Ces deux gènes, dont les produits sont indispensables à l'expression du phénotype cholinergique, pourraient donc être co-régulés. Les promoteurs de ces deux gènes ont été identifiés. Les mécanismes de régulation de l'expression de ces gènes sont actuellement étudiés. (©Elsevier, Paris)

choline acetyltransferase / vesicular acetylcholine transporter / cholinergic locus / regulatory mechanisms

1. Introduction

The expression of the cholinergic phenotype depends on the activity of a high affinity choline transporter as well as that of proteins involved in the synthesis, storage and release of the neurotransmitter acetylcholine (ACh). ACh is synthesized in the cytoplasm of presynaptic neurons by choline acetyltransferase (ChAT), then translocated into synaptic vesicles by the vesicular ACh transporter (VAcHT). cDNAs encoding ChAT have been cloned in different species which has led to the subsequent isolation of the ChAT gene (for review see [17]). The expression of this gene is complex. In rodent, the ChAT gene contains three 5' non-coding exons designated as R, N and M. The use of different promoters and alternative mRNA splicing within the 5' region gives rise to the synthesis of several mRNAs with different 5' untranslated sequences (*figure 1*) [10, 14]. Two promoters of the murine ChAT gene have been identified upstream from the exons R and M ([2, 9, 14]; Bejanin et al., unpublished results).

Analysis of the sequence of the first intron of the rat ChAT gene has revealed the presence of an open

reading frame of 1590 bp encoding the VAcHT protein and directed in the same transcriptional orientation as the ChAT gene (*figure 1*) [3]. This unique gene organization has been demonstrated in parallel in *Caenorhabditis elegans* and in man [1, 8] and more recently in mouse and *Drosophila* [11, 16]. The conservation of these gene arrangements between evolutionary separated species suggests its functional significance which may be the coordinated expression of the ChAT and VAcHT genes.

2. Transcription of the rat ChAT and VAcHT genes

The study of the regulatory mechanisms of these two embedded genes first requires the understanding of their mode of transcription. Are ChAT and VAcHT genes transcribed from independent promoters or from one common promoter region?

Northern blot analysis of rat VAcHT mRNAs has revealed their molecular diversity [3, 7]. Three mRNAs of 2.6, 3 and 3.9 kb have been detected in spinal cord and brainstem, and detailed analysis of VAcHT transcripts indicated that this diversity relies in their 5' non coding region (see below).

To test whether ChAT and VAcHT originate from the same transcription unit, RT-PCR experiments were performed with rat spinal cord mRNA using oligonucleotides complementary to the sequences of VAcHT and of the exon R. Two forms of VAcHT mRNAs were identified that contain the sequence of

* Present addresses: Department of Pathology, Kanazawa University School of Medicine, 13-1, Takaramachi, Kanazawa City, Ishikawa 920, Japan.

** École Normale Supérieure, 46, rue d'Ulm, 75005 Paris, France

*** Genset, Genomic Research Center, RN7, 91030 Evry cedex, France

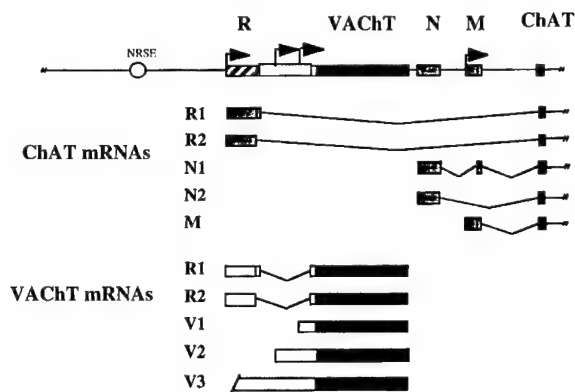


Figure 1. Schematic representation of the 5' region of the rat ChAT gene (top line) and the different ChAT and VAcHT mRNA species. Black boxes indicate coding sequences, gray and open boxes represent non-coding sequences of ChAT and VAcHT genes, respectively. Exon R, which is shared by both genes, is gray and white. Additional vertical bars represent splice sites. Arrows indicate the transcriptional start sites identified for the ChAT and VAcHT genes. The approximate position of the neuron restrictive silencer (NRSE) element is represented by a circle.

the exon R immediately followed by the sequence of an exon beginning at 308 bp upstream from the ATG translation initiation codon of VAcHT (*figure 1*) [3]. The existence of R-type VAcHT mRNAs suggests that ChAT and VAcHT genes can be transcribed from the same promoter region.

RT-PCR have also been carried out with primers complementary to VAcHT and to a sequence at 410 bp upstream from the ATG codon. The obtained amplification product revealed another type of VAcHT mRNA designated as V [3]. RNase protection experiments were then performed to localize the transcription initiation sites of the V-type VAcHT mRNAs [7]. Two clusters of start sites separated by about 450 bp have been identified for the VAcHT gene transcription and that give rise to two VAcHT mRNA designated as V1 and V2 (*figure 1*). Surprisingly, an additional VAcHT mRNA species, V3, has been identified whose sequence covers that of the first ChAT intron until at least 16 nucleotides from the 3' end of the exon R (*figure 1*). This mRNA may be produced from the same promoter as the R-type mRNAs.

The existence of V-type mRNAs has suggested the presence of VAcHT promoters within the first intron of the ChAT gene. Transient transfection experiments have revealed the presence of two different promoter regions within this intron that generate the V1 and V2 VAcHT transcripts [7].

Together, these data led us to propose the following model for the transcription of the rat ChAT/VAcHT gene locus. Both ChAT and VAcHT genes may be transcribed from a promoter region located upstream from the exon R. VAcHT mRNAs are generated when transcription is stopped at the polyadenylation site of the VAcHT gene and these mRNAs either are spliced to generate R-type mRNAs or remain unspliced (V3 mRNA). Alternatively, transcription may continue until the polyadenylation site of the ChAT gene giving rise to R-type ChAT mRNAs by removing a large fragment containing the VAcHT open reading frame. In addition, the VAcHT gene may be transcribed from the two promoters localized in the first intron of the ChAT gene to produce the VAcHT mRNA V1 and V2. Finally, the ChAT gene may be transcribed from two promoters located downstream from the VAcHT gene, thus producing the ChAT N- and M-type mRNAs.

3. Regulation of ChAT and VAcHT gene expression

The VAcHT promoter regions described above are highly active in non cholinergic cell lines [7]. Therefore, they lack the regulatory motifs responsible for the cell-specificity of the VAcHT gene expression. These motifs may be located upstream from the R-type promoter. Indeed, a rat genomic region upstream from the R exon has been shown to confer cholinergic specificity to the R-type promoter in vitro [9]. This same region directs the in vivo expression of a heterologous downstream promoter in cholinergic neurons [12]. Moreover, it contains a neuron restrictive silencer element (NRSE) that, when fused to a heterologous promoter, acts as a silencer in non-neuronal cells [13]. This raises the question of whether this region also restricts the expression of the VAcHT gene to cholinergic cells.

This question is being addressed by transient transfection experiments of different cell lines with constructs of the VAcHT promoters fused to the luciferase gene. These constructs contain the VAcHT promoters upstream and/or downstream from the exon R, that include or not the NRSE-containing region. Our results reveal that this region down regulates, but does not completely inhibit, the activity of the VAcHT promoters in non-neuronal cells. This suggests that other regulatory domains of the VAcHT gene are required to strictly restrict the expression of this gene to cholinergic cells. Mutation of the NRSE element will allow us to test its involvement in the regulation of the VAcHT gene transcription.

The proximity of the ChAT and VACHT genes, which encode different proteins required for the same neuronal phenotype, suggests the existence of regulatory mechanisms that coordinate their concomitant expression. To investigate this possibility, we ascertained that signals that induce the expression of the ChAT gene also induce that of the VACHT gene. Using cultured sympathetic neurons as an in vitro model, several classes of factors, such as the cholinergic differentiation factor/leukemia inhibitory factor (CDF/LIF) or retinoic acid (RA), have been shown to induce ChAT activity and to increase the level of the multiple ChAT mRNA species in these neurons ([6] and references cited therein). Both CDF/LIF and RA increased the amount of VACHT protein, parallelly and with comparable induction ranges to that of ChAT activity. Moreover, these two factors increased concomitantly VACHT and ChAT mRNA levels [4]. Similar findings have been reported by others [5, 15]. These data are consistent with the notion that transcription of both the ChAT and VACHT genes is subject to common regulatory mechanisms.

In order to identify the *cis*-active elements involved in the regulation of these genes by CDF/LIF and RA in cultured sympathetic neurons, it was necessary to transfect these neurons. The fact that classical transfection methods were ineffective led us to develop a protocol of microinjection of plasmids with reporter genes in these neurons, followed by the quantification and the normalization of the reporter gene activity. Reproducible reporter gene activities as well as induction of a CDF/LIF responsive promoter could be measured in microinjected neurons (Pajak et al., in preparation). This strategy is now used to identify the CDF/LIF and RA responsive elements of the ChAT and VACHT gene locus.

Acknowledgments

This work was supported by CNRS, INSERM, Rhône-Poulenc Rorer and the Institut de Recherche sur la Moelle Epiniere.

References

- [1] Alfonso A., Grundahl K., McManus J.R., Asbury J.M., Rand J.B., Alternative splicing leads to two cholinergic proteins in *Caenorhabditis elegans*, *J. Mol. Biol.* 241 (1994) 627–630.
- [2] Bejanin S., Habert E., Berrard S., Dumas Milne Edwards J.B., Loeffler J.B., Mallet J., Promoter elements of the rat choline acetyltransferase gene allowing nerve growth factor inducibility in transfected primary cultured cells, *J. Neurochem.* 58 (1992) 1580–1583.
- [3] Bejanin S., Cervini R., Mallet J., Berrard S., A unique gene organization for two cholinergic markers, choline acetyltransferase and a putative vesicular transporter of acetylcholine, *J. Biol. Chem.* 269 (1994) 21944–21947.
- [4] Berrard S., Varoqui H., Cervini R., Israël M., Mallet J., Diebler M.F., Coregulation of two embedded gene products, choline acetyltransferase and the vesicular acetylcholine transporter, *J. Neurochem.* 65 (1995) 939–942.
- [5] Berse B., Blusztajn J.K., Co-ordinated up-regulation of choline acetyltransferase and vesicular acetylcholine transporter gene expression by retinoic acid receptor alpha, cAMP, and CNTF/LIF signaling pathways in a murine septal cell line, *J. Biol. Chem.* 270 (1995) 22101–22104.
- [6] Cervini R., Berrard S., Bejanin S., Mallet J., Regulation by CDF/LIF and retinoic acid of multiple ChAT mRNAs produced from distinct promoters, *NeuroReport* 5 (1994) 1346–1348.
- [7] Cervini R., Houhou L., Pradat P.F., Bejanin S., Mallet J., Berrard S., Specific vesicular acetylcholine transporter promoters lie within the first intron of the rat choline acetyltransferase gene, *J. Biol. Chem.* 270 (1995) 24654–24657.
- [8] Erickson J.D., Varoqui H., Schäfer M.K., Modi W., Diebler M.F., Weihe E., Rand J., Eiden L.E., Bonner T.I., Usdin T.B., Functional identification of a vesicular acetylcholine transporter and its expression from a 'cholinergic' gene locus, *J. Biol. Chem.* 269 (1994) 21929–21932.
- [9] Ibanez C.F., Persson H., Localization of sequences determining cell type specificity and NGF responsiveness in the promoter region of the rat choline acetyltransferase gene, *Eur. J. Neurosci.* 3 (1991) 1309–1315.
- [10] Kengaku M., Misawa H., Deguchi T., Multiple mRNA species of choline acetyltransferase from rat spinal cord, *Mol. Brain Res.* 18 (1993) 71–76.
- [11] Kitamoto T., Wang W., Salvaterra P.H., Structure and organization of the *Drosophila* cholinergic locus, *J. Biol. Chem.* 273 (1998) 2706–2713.
- [12] Lönnerberg P., Lendahl V., Funakoshi H., Richter L., Persson H., Ibanez C.F., Regulatory region in choline acetyltransferase gene directs developmental and tissue-specific expression in transgenic mice, *Proc. Natl. Acad. Sci. USA* 92 (1995) 4046–4050.
- [13] Lönnerberg P., Schoenherr C.J., Anderson D.J., Ibanez C.F., Cell type-specific regulation of choline acetyltransferase gene expression, *J. Biol. Chem.* 271 (1996) 33358–33365.
- [14] Misawa H., Ishii K., Deguchi T., Gene expression of mouse choline acetyltransferase. Alternative splicing and identification of a highly active promoter region, *J. Biol. Chem.* 267 (1992) 20392–20399.
- [15] Misawa H., Takahashi R., Deguchi T., Coordinate expression of vesicular acetylcholine transporter and choline acetyltransferase in sympathetic superior cervical neurones, *NeuroReport* 6 (1995) 965–968.
- [16] Naciff J.M., Misawa H., Dedman J.R., Molecular characterization of the mouse vesicular acetylcholine transporter gene, *NeuroReport* 8 (1997) 3467–3473.
- [17] Wu D., Hersch L.B., Choline acetyltransferase: celebrating its fiftieth year, *J. Neurochem.* 62 (1994) 1653–1663.

Regulation of the cholinergic gene locus

Hiromitsu Tanaka, Masahito Shimojo, Donghai Wu, Louis B. Hersh

Department of Biochemistry, University of Kentucky, Lexington, Kentucky 40536-0084, USA

Abstract — DNase I hypersensitive site mapping of the human cholinergic gene locus has been used to detect cholinergic specific potential regulatory sites. Analysis of mutant PC12 cell lines provides evidence that protein kinase A II is required and coordinately regulates basal expression of both the ChAT and VACHT genes. (©Elsevier, Paris)

Résumé — **Régulation du locus génétique cholinergique** Nous avons utilisé la cartographie des sites hypersensibles à la DNase I dans le locus génétique cholinergique humain pour identifier des sites régulateurs potentiels. L'analyse de lignées mutantes de cellules PC12 indique que la protéine kinase A II est impliquée dans la régulation coordonnée de l'expression des gènes ChAT et VACHT. (©Elsevier, Paris)

choline acetyltransferase / vesicular acetylcholine transporter / transcriptional regulation / coordinate regulation / protein kinase A

1. Introduction

The cholinergic gene locus is comprised of the genes for the biosynthetic enzyme choline acetyltransferase (ChAT) and the gene for the vesicular acetylcholine transporter (VACHT). The VACHT gene, whose open reading frame is within a single exon, lies within the intron between the first and second exons of the ChAT gene. This unusual gene arrangement is conserved across such diverse species as the nematode, *Drosophila*, and mammals, and suggests potential coordinate regulation of the gene.

The mechanisms responsible for the transcriptional regulation of the cholinergic gene locus have been studied by both transient cell transfection analysis as well as through transgenic mice models. Based on studies from a number of laboratories a complex regulatory pattern at this locus is emerging. Recent transgenic mouse models [10, 13], each utilizing different non-overlapping regions of the locus, were reported to provide cell specific expression of the locus. This suggests the existence of multiple cell specific regulatory elements in the gene.

We report here two approaches to study regulation of the cholinergic gene locus. In one we analyzed the chromatin structure of the human cholinergic gene locus by DNase I hypersensitive mapping to identify potential regulatory sites in the gene. In the other we have used mutant PC12 cell lines to demonstrate coordinate regulation of the ChAT and VACHT genes by a protein kinase A dependent mechanism.

2. Materials and methods

2.1. Cell lines

The cell lines used in this study include CHP134, a cholinergic neuroblastoma cell line, HeLa, MCF-7, a human breast adenocarcinoma cell line, HeLa, PC12, and two PC12 mutant cell lines. PC12/A123.7 expresses a mutant regulatory PKA subunit [2, 5] and contains reduced levels of PKA I and PKA II. PC12/A126-1B2 was generated by nitrosoguanidine mutagenesis and lacks PKA II activity, but contains normal levels of PKA I [15].

2.2. DNase I hypersensitivity mapping

Cell nuclei were isolated from detergent lysed cells by centrifugation and resuspended in digestion buffer (10 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 0.5 mM CaCl₂, 25 mM KCl, 0.5 mM DTT and 0.35 M sucrose). Nuclei were digested with 1, 5, or 10 units of DNase I for 10 min at 25 °C. Following termination the sample was digested with proteinase K followed by phenol, phenol/chloroform, and chloroform extraction, and precipitation with ethanol. The DNA pellet was treated with RNase, followed by digestion with proteinase K, and extraction and precipitation as noted above. This DNA was digested with a suitable restriction enzyme and electrophoresed on a 0.8% agarose gel. The DNA was transferred to a Hybond N (+) membrane and hybridized with a probe radiolabeled by the random priming method [14]. The washed membrane was exposed to Kodak XAR-5 film.

2.3. Determination of choline acetyltransferase activity

Choline acetyltransferase was assayed by a modification of the method of Fonnum [4] as described by Hersh [6].

2.4. Competitive PCR

Total cellular RNA was isolated from cultured cells by the method of Chomczynski and Sacchi [1]. Single-stranded cDNAs were synthesized using Molony murine leukemia virus (M-MLV) reverse transcriptase using specific primers for VAcHT or ChAT. Single-stranded cDNA (0.1 µg) was amplified by PCR in a programmable thermal cycler that included variable amounts of an appropriate internal standard and a labeled nucleotide. Following amplification the PCR product was electrophoresed on a 5% polyacrylamide gel and autoradiographed. Signal intensities were quantified directly from the bands with a Storm[™] PhosphorImager and Image Quant.

2.5. Other materials

RNase inhibitor, dibutyryl cAMP, and deoxynucleotides were purchased from Boehringer Mannheim (Indianapolis, IN, USA). DNase I was from Promega (Madison, WI, USA). H-89 and H-9 dihydrochloride were obtained from Calbiochem (La Jolla, CA, USA). M-MLV reverse transcriptase and Taq DNA polymerase were purchased from Promega (Madison, WI). 8- (4-chlorophenylthio) adenosine-3',5'-cyclic monophosphate (8-CPT-cAMP), 8-piperidinoadenosine-3',5'-cyclic monophosphate (8-PIP-cAMP), 8- (4-aminohexyl) aminoadenosine-3',5'-cyclic monophosphate (8-AHA-cAMP), and N⁶-benzoyladenine-3',5'-cyclic monophosphate (N⁶-BNZ-cAMP) were purchased from BioLog Life Science Institute (Bremen, Germany). All other reagents were from Sigma Chem. Co. (St. Louis, MO, USA).

3. Results

DNase I hypersensitive site mapping was employed as a method to locate specific elements involved in the regulation of the human cholinergic gene locus. CHP134 cells were used as a cholinergic cell line and HeLa cells and MCF-7 cells as non-cholinergic cell lines. A variety of probes were employed to detect the DNase I dependent fragmentation pattern of nuclear DNA digested with restriction enzymes. This analysis led to the identification of eight DNase I hypersensitive sites in the cholinergic cell line (*figure 1*). In contrast the two non-cholinergic cell lines showed two DNase I hypersensitive sites (*figure 1*).

Analysis of the two DNase I hypersensitive sites seen in the inactive cholinergic gene from the non-cholinergic cell lines indicated that site 1' corresponds to the neuronal restrictive silencer element, NRSE, previously identified in the gene [11, 3]. The other site, site 6, corresponded to an enhancer element identified in this laboratory [9]. Of the eight DNase I hypersensitive sites found in active chromatin from CHP 134 cells six of these appear to correspond to core promoter regions, site 2, R promoter, site 3, VAcHT promoter, site 4, V1 VAcHT

promoter, site 7, N promoter, site 8, M promoter. In addition site 1 is a cholinergic specific site which resides in the vicinity of the NRSE, site 5 appears to be an enhancer element, while site 6 is the previously identified enhancer element noted above. These latter sites may be key regulators in the cell specific expression of this locus.

In a different approach to study regulation of the cholinergic gene locus we utilized two protein kinase A (PKA) mutant cell lines, A126-1B2 which is deficient in PKA II and A123.7 which has decreased levels of PKA I and PKA II. A PCR based assay was used to demonstrate that both ChAT and VAcHT mRNA levels in the mutant cell lines were significantly decreased (*figure 2*). Treatment of the parental PC12 cell line, but not the PKA mutants, with dbcAMP increased ChAT mRNA levels 3.6-fold and VAcHT mRNA levels 4.2-fold. Treatment of the parental PC12 cell line with two selective PKA inhibitors H-9 or H-89 reduced both ChAT and VAcHT mRNA levels to ~1/3 of that in the untreated cells (*figure 2*). The reduction of VAcHT mRNA again paralleled that of ChAT mRNA. The dbcAMP induced increase in ChAT mRNA was paralleled by a 5-fold increase in ChAT activity, and H89 blocked this increase. H89 and H9 alone decreased ChAT activity to 1/4 and 1/5 the control level, respectively.

The above results suggest that PKA II, but not PKA I, may regulate the cholinergic gene locus. We confirmed this by demonstrating 2.5 fold increase increases in ChAT activity produced by a combination of 8-CPT-cAMP and N⁶-BNZ-cAMP, which selectively activates PKA II, with no effect of a combination of 8-AHA-cAMP and 8-PIP-cAMP which selectively activates PKA I.

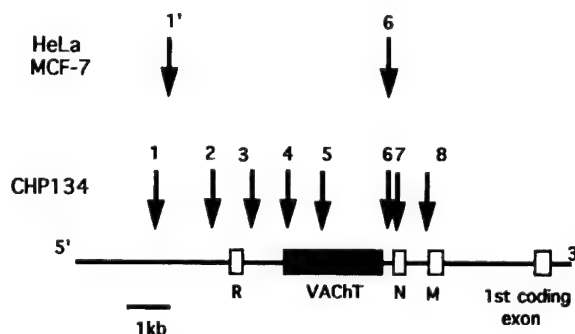


Figure 1. DNase I hypersensitive sites of the cholinergic gene locus in CHP134, HeLa and MCF-7 cells. The R, N, M, and first coding exons are illustrated as well as the VAcHT gene open reading frame. Arrows indicate the DNase I hypersensitive sites.

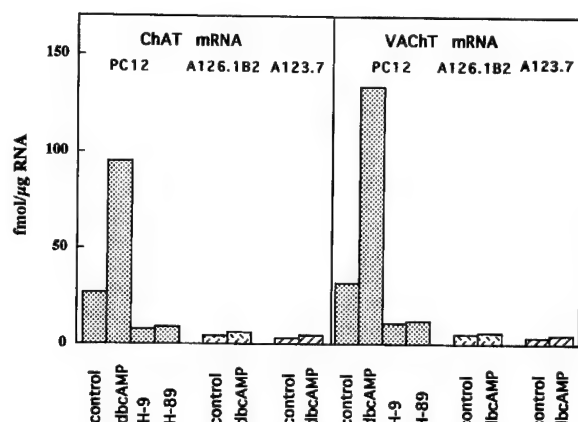


Figure 2. ChAT and VACHT mRNA levels in PC12 cells and PKA mutant cell lines.

4. Discussion

For many genes, it has been reported that the elements which correspond to DNase I hypersensitive sites play an important role for transcriptional regulation. We observed a number of cholinergic specific DNase I hypersensitive sites in regions of the gene which do not exhibit cell specific reporter gene activity [7]. It is possible that these sites are cholinergic cell specific as a result of the chromatin structure of the cholinergic gene locus.

An interesting finding is the presence of a DNase I hypersensitivity site, site 1, which is located more than 1 kb upstream of the R exon. This is the region of the cholinergic gene locus shown to contain a cholinergic specific repressor in the rat gene [8]. The presence of site-1' in non-cholinergic cells, which corresponds to the NRSE, is understandable since this element can repress expression of the cholinergic gene in non-neuronal cells [11]. However, the presence of site 6, which corresponds to an enhancer element, is unexpected. We suggest that this site might act in conjunction with the NRSE to silence this locus in non-cholinergic cells.

Studies with protein kinase A deficient PC12 cells establish the requirement for this kinase for basal expression of both the ChAT and VACHT genes. Furthermore this regulation appears to be coordinate with these two genes. In the parental PC12 cell line, dbcAMP induced both ChAT and VACHT mRNAs in a parallel fashion. Similarly a parallel reduction in ChAT and VACHT mRNA levels was produced by the PKA inhibitors H-89 and H-9. In the protein kinase A deficient cell lines considerably lower ChAT and VACHT mRNA levels were observed compared to the PC12 parental line. These findings de-

monstrate that both ChAT and VACHT gene transcription are regulated by protein kinase A, and that this regulation is coordinate. The finding that ChAT and VACHT mRNA levels are reduced in a cell line which contains wild type levels of PKA I, but is devoid of PKA II activity, and the ability of PKA II agonists, but not PKA I agonists, to increase ChAT activity shows that regulation of the cholinergic gene is through a PKA II signaling pathway. Transient transfection analysis indicates that protein kinase A can act through a site on the human ChAT gene upstream of the VACHT promoter. This is clearly a different site than the CRE-like element shown by Misawa et al. [12] to be localized between the M exon and the first coding exon. The mechanism by which PKA II regulates the cholinergic gene locus is currently under investigation.

Acknowledgments

We thank Dr. John Wagner, Cornell University, for providing the PC12 cell lines used in this study. This work was supported by grants from the NIH, NIA AG05893 and AG05144.

References

- [1] Chomczynski P., Sacchi N., Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, *Anal. Biochem.*, 162 (1987) 156-159.
- [2] Correll L.A., Woodford T.A., Corbin J.D., Mellon P.L., McKnight G.S., Functional characterization of cAMP-binding mutations in Type I protein kinase, *J. Biol. Chem.* 264, (1989) 16672-16678.
- [3] Erickson J.D., Weihe E., Schafer M.K.-M., Neale E., Williamson L., Bonner T.I., Tao-Cheng J.-H., Eiden L.E., The VACHT/ChAT 'cholinergic gene locus': new aspects of genetic and vesicular regulation of cholinergic function, in: Klein J., Löffelholz K., (Eds.), *Progress in Brain Research: Cholinergic Mechanisms: From Molecular Biology to Clinical Significance*, Elsevier, 109, (1996) 69-82.
- [4] Fonnum F., A rapid radiochemical method for the determination of choline acetyltransferase, *J. Neurochem.* 24 (1975) 407-409.
- [5] Ginty D., Glowacka D., De Frano D., Wagner J.A., Nerve growth factor-induced neuronal differentiation after dominant repression of both type I and type II cAMP-dependent protein kinase activities, *J. Biol. Chem.* 266 (1991) 15325-15333.
- [6] Hersh L.B., Induction of choline acetyltransferase in the neuroblastoma x glioma cell line NG108-15, *Neurochem. Res.* 17 (1992) 1063-1067.
- [7] Hersh L.B., Kong C.F., Sampson C., Mues G., Li Y.-P., Fisher A., Hilt D., Baetge E.E., Comparison of the promoter region of the human and porcine choline acetyltransferase

- genes: localization of an important enhancer region, *J. Neurochem.* 61 (1993) 306–314.
- [8] Ibanez C.F., Persson H., Localization of sequences determining cell type specificity and NGF responsiveness in the promoter region of the rat choline acetyltransferase gene, *Eur. J. Neurosci.* 3 (1991) 1309–1315.
- [9] Inoue H., Baetge E. E., Hersh L.B., Enhancer containing unusual GC box-like sequences on the human choline acetyltransferase gene, *Mol. Brain Res.* 20 (1993) 299–304.
- [10] Lonnerberg P., Lendahl U., Funakoshi H., Arhlund-Richter L., Persson H., Ibanez C.F., Regulatory region in choline acetyltransferase gene directs developmental and tissue-specific expression in transgenic mice, *Proc. Natl. Acad. Sci. USA* 92 (1995) 4046–4050.
- [11] Lonnerberg P., Schoenherr C.J., Anderson D.J., Ibanez C.F., Cell-type specific regulation of choline acetyltransferase gene expression: Role of the neuron-restrictive silencer element and cholinergic specific enhancer sequences, *J. Biol. Chem.* 271 (1996) 33358–33365.
- [12] Misawa H., Takahashi R., Deguchi T., Transcriptional regulation of choline acetyltransferase gene by cyclic AMP, *J. Neurochem.* 60 (1993) 1383–1387.
- [13] Naciff J.M., Misawa H., Dedman J.R., Molecular characterization of the mouse vesicular acetylcholine transporter gene, *Neuroreport* 8 (1997) 3467–3473.
- [14] Sambrook J., Fritsch E.F., Maniatis T., *Molecular Cloning: A Laboratory Manual*. 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
- [15] Van Buskirk R., Corcoran T., Wagner J.A., Clonal variants of PC12 pheochromocytoma cells with defects in cAMP-dependent protein kinases induce ornithine decarboxylase in response to nerve growth factor but not to adenosine agonists, *Mol. Cell. Biol.* 5 (1985) 1984–1992.

The Schwann cell at the neuromuscular junction

Jeanine Koenig^{a, b}, Sabine De La Porte^c, Jacqueline Chapron^{b, d}

^a*Institut de Myologie, Groupe Hospitalier Pitié-Salpêtrière, 47, boulevard de l'Hôpital, F-75651 Paris cedex 13, France*

^b*Laboratoire de Neurobiologie Cellulaire, Université de Bordeaux II, Avenue des Facultés, 33405 Talence cedex, France*

^c*Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS-UPR 9040, Avenue de la Terrasse, 91198 Gif-sur-Yvette cedex, France*

^d*URA-CNRS 1126, 33120 Arcachon, UMR 5807, 33405 Talence, France*

Abstract — Synapses obtained *in vitro* in a system of co-culture of muscle cells and neurons are of embryonic type. We prepared a monoclonal antibody (6.17) which recognizes a molecule synthesized by Schwann cells and used it to show that the main characteristics of maturity (decrease in number of synapses, appearance of junctional folds, and suppression of butyrylcholinesterase expression) are under the control of Schwann cells. In addition, Schwann cells have the capacity to aggregate the acetylcholine receptors in myotube cultures. (©Elsevier, Paris)

Résumé — La cellule de Schwann à la jonction neuromusculaire. Les synapses obtenues dans un système de co-cultures cellules musculaires-neurones sont de type embryonnaire. En utilisant un anticorps monoclonal obtenu au laboratoire (le 6.17), dirigé contre un antigène schwannien, nous avons montré que certains caractères de maturité (diminution du nombre de synapses, apparition de plis sous-neuraux et répression de l'expression de la butyrylcholinestérase) sont contrôlés par la cellule de Schwann. Enfin, la cellule de Schwann possède la capacité d'agréger les récepteurs à acétylcholine dans des myotubes en culture. (©Elsevier, Paris)

acetylcholinesterase / butyrylcholinesterase / acetylcholine receptor / neuromuscular junction / Schwann cells

Schwann cells wrap around nerve axons to provide electrical insulation and are essential for the formation, maintenance and repair of the neuromuscular junction (NMJ) [1]. At the NMJ, they are juxtaposed with nerve terminal and muscle cell, with which they interact during synaptogenesis. We focused on their role in maturation of the NMJ.

Establishment of an NMJ involves a sequence of complex mechanisms. The progression of events, from the first neuromuscular contact (14–15 embryonic days) to the mature synapse (15 days post-natal), occurs over 3 weeks. As soon as the first nerve-muscle contact is established, acetylcholine receptors (AChR) and cholinesterases (acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)) accumulate at synaptic sites. The accumulation of AChR in the muscular membrane is a prerequisite for normal accumulation of AChE in the basal lamina of the synaptic cleft: in different systems of cultures, the conditions which prevented clustering of AChR (anti-AChR antibodies, deficiency in AChR of the variant C2 cell line) also suppressed AChE clustering [5]. AChR mature up to 2 weeks after birth under the control of molecules synthesized by the motoneuron (reviewed in [8, 14]). AChR consists of α , β , γ , and δ subunits at the embryonic stage, and the γ subunit is replaced by the ϵ subunit 2–3 days after innervation. This substitution affects the channel properties of the AChR (conductance and open time). Two molecules synthesized by the motoneuron are known to induce expression of the ϵ gene: ace-

tylcholine receptor-inducing activity (ARIA)/heregulin and agrin. Another neuronal molecule, calcitonin-gene related peptide (CGRP), also increases transcription of the AChR α -subunit gene, but not of other subunits. Agrin also induces the clustering of AChR, AChE, BChE and heparan sulphate proteoglycan (HSPG), a component of the basal lamina.

Cholinesterases exist in several oligomeric molecular forms, distinguished by their sedimentation coefficient: the globular and the asymmetric forms (for review see [12]). The globular forms correspond to the monomer G1, dimer G2 and tetramer G4, and the asymmetric (A) forms to one, two or three tetramers linked to a collagen-like tail (A4, A8 and A12, respectively). The catalytic subunits corresponding to AChE and BChE are encoded by distinct genes. The role of AChE in cholinergic transmission is unambiguous, but the function of BChE is not clear, except as an adhesion molecule [11]. BChE, which is little expressed in the adult NMJ, also hydrolyses the neurotransmitter acetylcholine, and may thus participate in neurotransmission *in vitro*, and *in vivo* during development, and to a lesser degree in the adult NMJ. During development of chicken synapses, Tsim et al. [15] have demonstrated the transition of heterogeneous collagen-tailed forms, containing both AChE and BChE subunits, to homogeneous AChE collagen-tailed molecules. Berman et al. [2] showed that, in whole rat skeletal muscle (i.e., without distinguishing synaptic and extrasynaptic zones), there is a gradual increase in

muscle AChE activity, and a concomitant decrease in BChE activity, between 7 and 15 days after birth. We demonstrated that AChE and BChE are initially present in equal amounts at the synapses, but BChE levels diminish sharply between 7 and 15 days after birth [4], the stage at which the synaptic Schwann cell membrane becomes juxtaposed with the muscle membrane [6].

Synapses obtained *in vitro*, by conventional techniques of contact between myoblasts and spinal cord neurons, have the morphological, biochemical and electrophysiological characteristics of embryonic synapses. The events of postnatal maturation, such as disappearance of polyinnervation, changes in levels of AChE and BChE, and appearance of junctional folds, have never been observed, whatever the duration of culture or the age (embryonic or adult) of the cells used. In co-cultures of adult human muscles with explants from whole cord and dorsal root ganglia of 13- to 14-day-old rat embryos, Kobayashi et al. [9] observed the formation of mature muscle fibers, as characterized by contractions and striations, well-organized AChE clusters, and the trend from multifocal to unifocal innervation. This result suggested that Schwann cells of dorsal root ganglia participate in synaptic maturation.

The factors involved in the morphological maturation (disappearance of polyinnervation, appearance of junctional folds) and changes of synaptic cholinesterases were not identified and we postulated that Schwann cells were implicated in these events. We prepared mixed cultures, combining muscle cells (from the hindleg of 18-day-old rat embryos), spinal cord cells (from 14-day-old embryos) and Schwann cells (from the sciatic nerve of new-born animals or from the Schwann cell line TSC2, a gift from Prof. Tennekoon, Philadelphia, USA). Schwann cells and spinal cord cells were added to the muscle cells at day 5 of culture, the stage at which the myotubes were formed, contracting and morphologically differentiated. We prepared a monoclonal antibody (6.17) which recognizes a molecule synthesized by Schwann cells and is co-localized with the AChR at the NMJ. 6.17 antigen appears to be localized in the synaptic space, but not in the basal lamina: on cryostat sections of adult rat lens, 6.17 antibody labels the membranes of the lens cells but not the basal lamina. *In vivo* in the rat, 6.17 antigen is initially distributed diffusely and concentrates at the synapse 15 days after birth, and its maintenance at the NMJ depends on innervation: antigen 6.17 is present in the endomysium in the extrasynaptic zones of muscle fibers during maturation of the muscle and after denervation [10].

We have shown that during *in vitro* synaptogenesis, the addition of Schwann cells to muscle-neuron

co-cultures: 1) allows visualization of antigen 6.17 at the synapse and induces synaptic maturation (decrease in number of synapses (50%) and appearance of junctional folds) [3]; and 2) induces the disappearance of BChE at synaptic clusters, leaving only AChE activity, as in the adult neuromuscular junction [4]. This maturation is inhibited by the presence of antibody 6.17. Antigen 6.17 is secreted by Schwann cells and TSC2 cells, since medium conditioned by these cells reproduced the effect of the cells themselves: only AChE patches could be observed in the co-cultures in the presence of conditioned medium. 6.17 antibody inhibited the effect of Schwann cells and the effect of medium conditioned by these cells and TSC2 cells. It may be wondered whether antigen 6.17 corresponds to a previously characterized molecule [4], and its molecular characterization is in progress (in collaboration with H. Chneiweiss, INSERM U114, Paris). These results prompted us to postulate that Schwann cells could mediate changes in synaptic maturation during development. Heregulins and their specific receptors, members of the *erbB* family of tyrosine kinases,

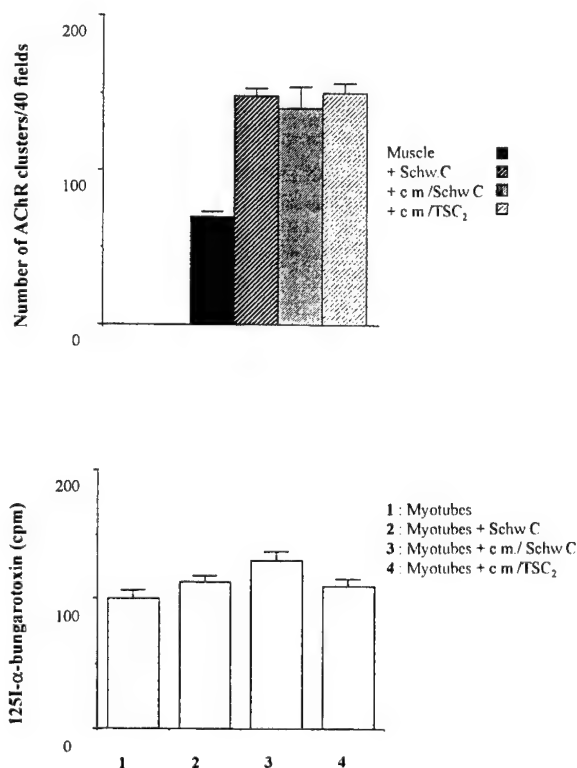


Figure 1. Number of AChR clusters (top) and ¹²⁵I-α-bungarotoxin binding sites (bottom) in 7-day-old myotubes cultured for 2 days with Schwann cells or with conditioned medium (c.m.) by Schwann or TSC2 cells.

have been implicated in the control of growth and development of Schwann cells. Homozygous *erbB3* mutant embryos lack Schwann-cell precursors and Schwann cells that accompany peripheral axons of motor (and sensory) neurons [13]. It will be of interest to look at the possible presence of high levels of BChE in such mutants.

AChR clustering may be induced by neuronal agrin, electric fields, latex beads, and basal lamina components (collagen, laminin) (for review see [7]). As the Schwann cell is closely associated with the nerve terminal, we investigated whether it plays a role in the formation of AChR clusters, and found that it does. When primary Schwann cells or medium conditioned by Schwann cells or by TSC2 cells were added to muscle cells in culture, the number of AChR sites increased by only 10%, but the number of AChR clusters increased by 100% (*figure 1*). Antibody 6.17 does not inhibit the Schwann cell-mediated increase in the number of AChR clusters. This strengthens the idea that antigen 617 is specifically implicated in synaptic maturation. We have tested the possibility that agrin synthesized by the Schwann cell is the molecule responsible for this increase. But the agrin isoform expressed by the Schwann cell is the inactive one (B0) (in collaboration with G. Escher, Institut d'Embryologie, Lausanne, Switzerland). We are currently testing different factors synthesized and released by Schwann cells.

Acknowledgments

This work was supported by grants from the CNRS, University of Bordeaux, and the Association Française contre les Myopathies (AFM).

References

- [1] Balice-Gordon R.J., Dynamic roles at the neuromuscular junction, *Curr. Biol.* 6 (1996) 1054–1056.
- [2] Berman H.A., Decker M.M., Sangme J., Reciprocal regulation of acetylcholinesterase and butyrylcholinesterase in mammalian skeletal muscle, *Dev. Biol.* 120 (1987) 154–161.
- [3] Chapron J., Koenig J., In vitro synaptic maturation, *Neurosci. Lett.* 106 (1989) 19–22.
- [4] Chapron J., De La Porte S., Delépine L., Koenig J., Schwann cells modify expression of acetylcholinesterase and butyrylcholinesterase at rat neuromuscular junctions, *Eur. J. Neurosci.* 9 (1997) 260–270.
- [5] De La Porte S., Chaubourt E., Fabre F., Poulas K., Chapron J., Eymard B., Tzartos S., Koenig J., Accumulation of acetylcholine receptors is a necessary condition for normal accumulation of acetylcholinesterase during in vitro neuromuscular synaptogenesis, *Eur. J. Neurosci.* 10 (1998) 1631–1643.
- [6] Desaky J., Uehara Y., Formation and maturation of subneural apparatus at neuromuscular junctions in post-natal rats. A scanning and transmission electron microscopic study, *Dev. Biol.* 199 (1987) 390–401.
- [7] Duclert E., Changeux J.P., Acetylcholine receptor gene expression at the developing neuromuscular junction, *Physiol. Rev.* 75 (1995) 339–368.
- [8] Hall Z.W., Sanes J.S., Synaptic structure and development: the neuromuscular junction, *Cell* 72, *Neuron* 10 (1993) 99–121.
- [9] Kobayashi T., Askanas V., Engel K., Human muscle cultured in monolayer and co-cultured with fetal rat spinal cord: importance of dorsal rat ganglia for achieving successful functional innervation, *J. Neurosci.* 7 (1987) 3131–3141.
- [10] Koenig J., Chapron J., Vigny M., Does the Schwann cell synthesize a molecule concentrated at the neuromuscular junction?, *Neurosci. Lett.* 89 (1988) 265–270.
- [11] Layer P.G., Willbold E., Cholinesterases in avian neurogenesis, *Int. Rev. Cytol.* 151 (1994) 139–181.
- [12] Massoulié J., Pezzementi L., Bon S., Krejci E., Vallette F.M., Molecular and cellular biology of cholinesterases, *Prog. Neurobiol.* 41 (1993) 31–91.
- [13] Riethmacher D., Sonnenberg-Riethmacher E., Brinkmann V., Yamaai T., Lewin G.R., Birchmeier C., Severe neuropathies in mice with targeted mutations in the *ErbB3* receptor, *Nature* 389 (1997) 725–730.
- [14] Sanes J.R., Genetic analysis of postsynaptic differentiation at the vertebrate neuromuscular junction, *Curr. Opin. Neurobiol.* 7 (1997) 93–100.
- [15] Tsim K.W.K., Randall W.R., Barnard E.A., Synaptic acetylcholinesterase of chicken muscle changes during development from a hybrid to a homogeneous enzyme, *EMBO J.* 7 (1988) 2451–2456.

Lipid peroxidation and changes in cytochrome c oxidase and xanthine oxidase activity in organophosphorus anticholinesterase induced myopathy

Zhen P. Yang, Wolf-D. Dettbarn*

Department of Pharmacology and Neurology, Vanderbilt University, School of Medicine, Nashville, TN 37212, USA

Abstract — A possible role of radical oxygen species (ROS) initiated lipid peroxidation in diisopropylphosphorofluoridate (DFP)-induced muscle necrosis was investigated by quantifying muscle changes in F₂-isoprostanes, novel and extremely accurate markers of lipid peroxidation in vivo. A significant increase in F₂-isoprostanes of 56% was found in the diaphragm of rats 60 min after DFP-induced fasciculations. As possible source of ROS initiating lipid peroxidation, the cytochrome-c oxidase (Cyt-ox) and xanthine dehydrogenase-xanthine oxidase (XD-XO) systems were investigated. Within 30 min of onset of fasciculations Cyt-ox activity was reduced by 50% from 0.526 to 0.263 $\mu\text{mol/mg prot/min}$ and XO activity increased from 0.242 to 0.541 $\mu\text{mol/mg prot/min}$. Total XD-XO activity was unchanged, indicating a conversion from XD into XO. In rats pretreatment with the neuromuscular blocking agent d-tubocurarine, prevented DFP-induced fasciculations, increases in F₂-isoprostanes and changes in Cyt-ox or XD-XO. The decrease in Cyt-ox and increase in XO suggest that ROS are produced during DFP induced muscle fasciculations initiating lipid peroxidation and subsequent myopathy. (©Elsevier, Paris)

Résumé — Peroxydation des lipides et modification de la cytochrome C oxydase et de la xanthine oxydase dans la myopathie induite par les inhibiteurs anticholinestérasiques organophosphorés. Nous avons examiné le rôle possible de la peroxydation de lipides par des résidus libres de l'oxygène (ROS) dans la nécrose musculaire induite par le diisopropylphosphorofluoridate (DFP), en quantifiant le niveau d'isoprostane F₂ musculaire, qui constitue un nouveau marqueur, extrêmement précis, de la peroxydation lipidique in vivo. Nous avons trouvé des augmentations significatives d'isoprostane F₂ (56%) dans le diaphragme de rat, 1 heure après l'induction de fasciculations par le DFP. La cytochrome C oxydase (Cyt-ox) et la xanthine déshydrogénase-xanthine oxydase (XD-XO) peuvent produire des radicaux libres (ROS) entraînant la peroxydation des lipides. En 30 minutes après le début des fasciculations, l'activité de la Cyt-ox était réduite de 50% (de 0,526 à 0,263 $\mu\text{mole/mg de protéine/min}$) et l'activité de la XO était augmentée de 0,242 à 0,541 $\mu\text{mole/mg de protéine/min}$. L'activité XD-XO totale n'était pas modifiée, ce qui indique une conversion de XD en XO. Chez les rats, un pré-traitement par la d-tubocurarine bloque l'activité musculaire et les fasciculations induites par le DFP, et empêche l'augmentation d'isoprostane F₂ et les changements de l'activité Cyt-Ox et du système XD-XO. Nous suggérons que l'hyperactivité cholinergique induite par le DFP provoque l'apparition de radicaux libres et la peroxydation des lipides. La diminution de Cyt-Ox et l'augmentation de XO suggèrent que les ROS sont produits pendant les fasciculations et que la peroxydation des lipides qui en résulte est responsable de la myopathie induite par le DFP. (©Elsevier, Paris)

lipid peroxidation / free radicals / myopathy / muscle hyperactivity

1. Introduction

Systemic injection of non-lethal concentrations of organophosphorus anticholinesterases (OP-antiChE) causes neuromuscular injury consequent to induced sustained muscle fasciculations [10]. The chain of events linking prolonged acetylcholine (ACh) receptor activation to neuromuscular pathology is complex and not fully understood. Recent evidence supports the assumption that over-production of reactive oxygen species (ROS) and lipid peroxidation may have an important role in initiation of the neuromuscular lesions [20, 21].

In skeletal muscle, several sources of ROS have been identified, such as mitochondrial electron trans-

port, oxidases in sarcoplasmic reticulum and sarcolemma and cytosolic xanthine oxidase.

During normal conditions ROS, such as superoxide anion, hydrogen peroxide and hydroxyl radicals, are generated at a low rate by cytochrome c oxidase (Cyt-ox) and can be taken care of by the scavenger and antioxidant systems. However, a reduced capacity of this enzyme can lead to incomplete reduction of oxygen and thus increased ROS production [18], which may exceed the capacity of the cellular defence system.

Another source of free radicals is xanthine oxidase (XO). This enzyme exist as NAD⁺ dependent dehydrogenase (XD, EC1.1.1.2.0.4) and is converted into xanthine oxidase (EC1.1.3.2.2). Xanthine oxidase directly transfers electrons from the oxidation of hypoxanthine to molecular oxygen, producing ROS such as superoxide and hydrogen peroxide; both of these are very toxic and can interact with

* Correspondence and reprints

free fatty acids such as arachidonic acid which via prostaglandin synthesis generates more ROS [4, 6]. Conversion from XD to XO has been shown to occur during muscle ischemia [6], seizure activity [2] and muscle activity [17]. Previous studies have shown that a decrease in the endogenous antioxidant glutathione by buthionine sulfoxime (BSO) increased the DFP-induced lipid peroxidation and the severity of the myopathy [20].

The present investigation was undertaken to determine through appropriate measurements whether Cyt-ox, XD and XO activities are changed in muscle taken from DFP-treated rats. To rule out direct effects of DFP on these enzymes, the neuromuscular blocking agent d-tubocurarine was used in another series of experiments to prevent DFP-induced muscle hyperactivity.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 200–250 g were maintained on a 12 h light/dark cycle with food and tap water available ad libitum. For biochemical studies rats were decapitated 30–120 min following the DFP administration (0.5–1.7 mg/kg, s.c.). d-Tubocurarine (0.07 mg/kg) was given i.p. 30 min prior to the DFP injection and immediately after the DFP injection. Buthionine sulfoxime (BSO) 900 mg/kg, i.p. was given 3.5 and 1.5 h before DFP. For histochemistry rats were decapitated 24 h after the start of the experiment.

2.2. Enzyme determination

Cyt-ox activity was determined by measuring spectrophotometrically the oxidation rate of ferrocytochrome c at the absorbance of its α -band, 550 nm, as described by Wharton and Tzagoloff [19]. 0.1 M 2-(N-morpholine) ethanesulfonic acid (MES, pH 6.0) with 10 μ M EDTA was used as buffer. XO or XD activity was determined spectrophotometrically based on the production of uric acid at 295 nm [15]. XO activity was assayed in the absence of NAD⁺, and the XD-XO was measured in the presence of NAD⁺.

2.3. Lipid peroxidation

A series of prostaglandin F₂-like compounds termed F₂-isoprostanes are formed in vivo from the free radical-catalyzed peroxidation of arachidonic acid and are useful markers of oxidant stress [13]. We have used these F₂-isoprostanes as an index of lipid peroxidation.

2.4. Statistical evaluation

Student's *t*-test was used to establish significance at *P* < 0.05.

3. Results

3.1. DFP induced lipid peroxidation and muscle necrosis

Rats treated with DFP (1.5–1.7 mg/kg, s.c.) developed fasciculations within 10 min lasting for 4–8 h. Histochemical studies revealed necrotic lesions involving endplates. The number of necrotic lesions was reduced significantly when rats were pretreated with d-tubocurarine in order to prevent fasciculations (table I). Glutathione (GSH), an endogenous antioxidant, protects against oxidative stress in vivo, its depletion should increase lipid peroxidation and the number of necrotic fibers in our experiments. In rats when pretreated with BSO 3.5 and 1 h before DFP, the number of necrotic fibers was significantly higher than seen with DFP only (table I). The combined treatment of BSO and DFP reduced GSH from 1.36 ± 0.20 (100%) to 0.39 ± 0.11 (28%) μ mol/g muscle.

Significant increases of F₂-isoprostanes were detected following exposure of rats to 1.7 mg/kg over 30, 60 and 120 min. The largest increase of 56% from control 1.66 ± 0.20 ng/g tissue in F₂-isoprostanes was observed in rats treated with 1.7 mg/kg DFP following 60 min of exposure (*P* < 0.01). Fasciculations also were detected under these conditions. Lower doses of DFP, such as 1 mg/kg, which did not induce fasciculations, produced no increases in

Table I. Modification of DFP myopathy by pretreatment with d-tubocurarine or BSO.

	Treatment ^a			
	Control	DFP	d-Tubocurarine + DFP	BSO + DFP
Fasciculations	0	++++	0	++++
Number of necrotic fibers/1000 muscle fibers	2 ± 2 (0.2%)	479 ± 45^b (47.9%)	$73 \pm 19^{c,d}$	$655 \pm 67^{b,c}$ (65.5%)

^a Treatment: DFP (1.7 mg/kg), d-tubocurarine (0.07 mg/kg), BSO (900 mg/kg). All drugs were given i.p. with the exception of DFP which was given s.c. Rats were killed 24 h after DFP treatment for histochemistry. Values are the mean \pm S.E.M. of seven muscles. ^b*P* < 0.001 and ^c*P* < 0.01, between control and treated rats; ^d*P* < 0.01 and ^e*P* < 0.05 between DFP-only-treated rats and the rats with drug pretreatments.

formation of F₂-isoprostanes when compared to control animals.

Pretreatment with the neuromuscular blocking agent d-tubocurarine prevented the muscle hyperactivity and increases in F₂-isoprostanes formation otherwise elevated by DFP treatment.

3.2. Sources for ROS production-cytochrome c oxidase reduction

In diaphragm, following DFP, Cyt-ox was transiently reduced to 51% of control activity after 15 min ($P < 0.001$) and to 77% within 30 min ($P < 0.01$). By 60 min, the Cyt-ox activity had completely recovered (figure 1). The 0.5 mg/kg DFP treatment did neither cause muscle hyperactivity such as fasciculations, nor any significant Cyt-ox reduction (figure 1), myopathic lesions or increases in lipid peroxidation.

As shown in figure 2, following pretreatment with d-tubocurarine (0.07 mg/kg), only a 7.6% decrease in Cyt-ox activity was seen within 15 min following DFP (1.7 mg/kg). This is a significant protection of Cyt-ox activity when compared to the 51% reduction seen after DFP alone. Under these conditions, muscle fasciculations, increases in F₂-isoprostanes as well as the muscle necrosis were prevented.

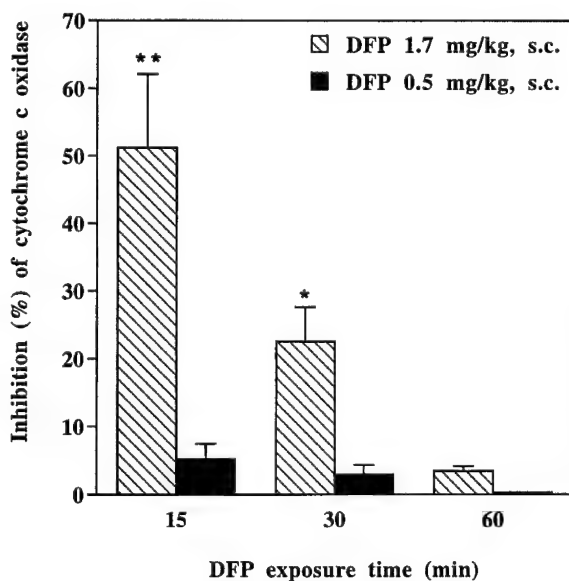


Figure 1. Reduction (%) of cytochrome c oxidase in rat diaphragm following various in vivo DFP treatments. Control activity of cytochrome c oxidase: 0.526 ± 0.093 $\mu\text{mol/min/mg protein}$. * $P < 0.01$; ** $P < 0.001$.

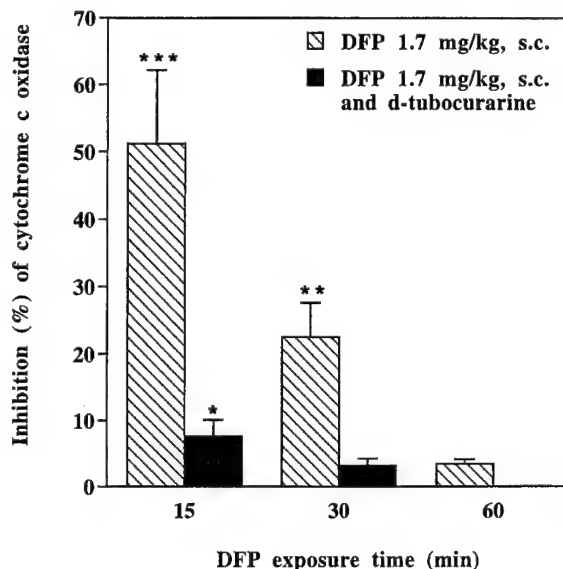


Figure 2. Effect of d-tubocurarine pretreatment on the cytochrome c oxidase reduction in rat diaphragm following various in vivo DFP treatments. d-Tubocurarine (0.07 mg/kg, i.p.) was given twice: 30 min before DFP injection and immediately after DFP injection. Control activity of cytochrome c oxidase was: 0.526 ± 0.093 $\mu\text{mol/min/mg protein}$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.3. Xanthine dehydrogenase/xanthine oxidase conversion

Within 30 min of DFP-induced fasciculations, an increase of XO activity was seen. During DFP-induced fasciculations, however, XO activity rose to 70% of the total XD + XO activity (table II). This increase in XO activity appears to have resulted from conversion of XD to XO, since the total activity of XD + XO remained constant throughout the 60 min observation period. Prevention of muscle hyperactivity through pretreatment with d-tubocurarine inhibited the conversion of XD to XO otherwise seen with DFP-induced muscle fasciculations (table II). These findings rule out a direct effect of DFP on Cyt-ox or XD-XO.

4. Discussion

Radical oxygen species are considered to be active mediators in the hyperactivity-induced skeletal muscle damage and inflammation, and in various muscle diseases [17]. While the potential sources of

Table II. Conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO) in rat diaphragm following in vivo DFP treatments, with or without the d-tubocurarine pretreatment^a.

	Control	DFP 30 min	DFP 60 min	DFP 30 min + d-tubocurarine	DFP 60 min + d-tubocurarine
XO ^b	0.242 ± 0.05	0.541 ± 0.13 ^d	0.553 ± 0.09 ^d	0.264 ± 0.07	0.236 ± 0.07
XD+XO ^c	0.794 ± 0.18	0.768 ± 0.20	0.803 ± 0.14	0.771 ± 0.22	0.825 ± 0.21
XO/XD	0.44	2.39 ^d	2.21 ^d	0.52	0.40
Increase of XO/XD from control (%)	—	443	402	18	

^aDFP was given 1.7 mg/kg, s.c. Rats were killed after 30 or 60 min of exposure to DFP. The drug pretreatment was same as in table I. ^bXO, μmol/min/mg protein. ^cXO + XD: μmol/min/mg protein. Values were mean ± S.D. of five rats.

^dP < 0.001 between control and treated animals.

ROS in muscle are numerous, such as mitochondrial electron transport, oxidases in sarcoplasmic reticulum and sarcolemma and cytosolic xanthine oxidase, the mechanisms that control the reactive oxidant production and the sites at which oxidative stress causes dysfunction remain little understood.

The present data demonstrate that DFP-induced muscle hyperactivity results in a rapid, significant reduction in the activity of Cyt-ox (figure 1) and an increased conversion of XD to XO (table II), suggesting the generation of ROS. This agrees with previous observations of increased levels of ROS following exercise-induced muscle damage [1, 5, 9, 14, 16].

Cytochrome c oxidase is the terminal complex in the mitochondria respiratory chain, which generates ATP by oxidative phosphorylation. During intensive muscle activity, the activity of Cyt-ox is reduced [7, 18], leading to an increase in the electron pressure within the electron transport chain and to the ROS production.

The significant increase in the ratio XO/XD following the onset of DFP-induced muscle fasciculations appear to be due to a conversion from XD to XO as no significant changes in the total activity (XO + XD) were observed. The shift of XD to XO begins with the onset of fasciculations and is still present after 60 min. As previously observed, DFP-induced fasciculations caused a rapid fall in ATP [8], leading to increased accumulation of Ca²⁺ [11]. The increased calcium may initiate the conversion of XD to XO through a calcium-dependent protease [3]. The loss of ATP is followed by an increase in ADP, which is further metabolized to adenosine, inosine and hypoxanthine, a substrate for XO [12]. Xanthine oxidase through the oxidation of hypoxanthine produces ROS as superoxide and hydrogen peroxide leading to lipid peroxidation and membrane damage.

A greatly increased rate of free radical production may exceed the capacity of the cellular defence sys-

tem such as antioxidants and permit a substantial attack of free radicals on lipids in the cell membrane causing lipid peroxidation leading to cell injury. This suggestion is supported by findings that BSO induced loss of GHS increased lipid peroxidation and the severity of the myopathy due to DFP [20].

The prevention of the DFP-induced muscle hyperactivity through the neuromuscular blocking agent d-tubocurarine attenuated the increase of F₂-isoprostanes [20, 21], prevented the change in Cyt-ox (figures 1, 2) and the conversion of XD into XO (table II). These data suggest that free radical generation during DFP-induced fasciculation is associated with AChE inhibition by DFP, and is not due to direct effects of DFP on Cyt-ox and XD/XO.

No studies to date have reported a relationship between OP-antiChE-induced muscle hyperactivity, lipid peroxidation, and muscle lesions. From the present studies, however, it is concluded that the accumulation of oxygen free radicals and the related increases in lipid peroxidation are caused by the DFP-induced muscle hyperactivity and that lipid peroxidation is a contributing initial factor in the mechanism of OP-antiChE-induced cell injury.

In summary, the results of this study demonstrated: 1) that the OP-antiChE, DFP, caused fasciculations leading to lipid peroxidation and muscle necrosis; 2) prevention of DFP-induced muscle hyperactivity by d-tubocurarine inhibits lipid peroxidation and necrosis and rules out a direct effect of DFP on Cyt-ox and XD-XO and thus support the hypothesis that OP-antiChE-induced muscle hyperactivity stimulates ROS production, through changes in cytochrome c oxidase and xanthine oxidase activity.

Acknowledgment

This work was supported by NIH grant RO1 ES04597.

References

- [1] Alessio H.M., Goldfarb A.H., Cutler R.G., MDA content increases in fast and slow twitch skeletal muscle with high intensity of exercise in rat, *Am. J. Physiol.* 255 (1988) C874-C877.
- [2] Battelli M.G., Buonomici L., Abbondanza A., Virgili M., Contestabile A., Stirpe F., Excitotoxic increase of xanthine dehydrogenase and xanthine oxidase in rat olfactory cortex, *Dev. Brain Res.* 86 (1995) 340-344.
- [3] Bindoli A., Cavallini L., Rigobello M.P., Coassin M.G., Dilisa F., Modification of the xanthine-converting enzyme of perfused rat heart during ischemia and oxidative stress, *Free Rad. Biol. Med.* 4 (1988) 163-167.
- [4] Bratell S., Folmerz P., Hansson R., Jonsson O., Lundstam S., Pettersson B.R., Schersten T., Effects of oxygen free radical scavengers, xanthine oxidase inhibition and calcium entry blockers on leaking of albumin after ischemia: an experimental study in rabbit kidneys, *Acta Physiol. Scand.* 134 (1988) 35-41.
- [5] Davies K.J.A., Quintanilha A.T., Brooks G.A., Packer L., Free radicals and tissue damage produced by exercise, *Biochem. Biophys. Res. Commun.* 107 (1982) 1198-1205.
- [6] Downey J.M., Hearse D.J., Yellon D.M., The role of xanthine oxidase during myocardial ischemia in several species including man, *J. Mol. Cell Cardiol.* 20 (1988) 55-63.
- [7] Gollnick P.D., Bertocci L.A., Kelso T.B., Witt E.H., Hodgson D.R., The effect of high intensity exercise on the respiratory capacity of skeletal muscle, *Pflügers Arch. (Eur. J. Physiol.)* 415 (1990) 407-413.
- [8] Gupta R.C., Dettbarn W.-D., Alterations of high-energy phosphate compounds in the skeletal muscles of rats intoxicated with diisopropylphosphorofluoridate (DFP) and soman, *Fund. Appl. Toxicol.* 8 (1987) 400-407.
- [9] Jenkins R.R., Free radical chemistry: relationship to exercise, *Sport Med.* 5 (1988) 156-170.
- [10] Laskowski M.B., Olson W.H., Dettbarn W.-D., Initial ultrastructural abnormalities at the motor endplate produced by a cholinesterase inhibitor, *Exp. Neurol.* 57 (1977) 94-100.
- [11] Leonard J.P., Salpeter M.M., Agonist-induced myopathy at the neuromuscular junction is mediated by calcium, *Cell Biol.* 82 (1979) 811-819.
- [12] McCord J.M., Free radicals and myocardial ischemia overview and outlook, *Free Rad. Biol. Med.* 4 (1988) 9-19.
- [13] Morrow J.D., Hill K.E., Burk R.F., Nammour T.M., Badr K.F., Roberts L.J., A series of F₂-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism, *Proc. Natl. Acad. Sci. USA* 87 (1990) 9383-9387.
- [14] Packer L., Vitamin E, physical exercise and tissue damage in animals, *Med. Biol.* 62 (1984) 105-109.
- [15] Parks D.A., Williams T.K., Beckman J.S., conversion of xanthine dehydrogenase to oxidase in ischemic rat intestine: a reevaluation, *Am. J. Physiol.* 254 (Gastroint. Liver Physiol. 17) (1988) G768-774.
- [16] Radak Z., Asano K., Inoue M., Kizaki T., Oh-Ishi S., Suzuki K., Taniguchi N., Ihno H., Superoxide dismutase derivative reduces oxidative damage in skeletal muscle of rats during exhaustive exercise, *J. Appl. Physiol.* 79 (1995) 129-135.
- [17] Sjodin B., Westing Y.H., Apple F.S., Biochemical mechanisms for oxygen free radical formation during exercise, *Sports Med.* 10 (1990) 236-254.
- [18] Soussi B., Idstrom J.-P., Schersten T., Bylund-Fellenius A.-C., Cytochrome c oxidase and cardiolipin alterations in response to skeletal muscle ischemia and reperfusion, *Acta Physiol. Scand.* 138 (1990) 107-114.
- [19] Wharton D.C., Tzagoloff A., Cytochrome oxidase from beef heart mitochondria, *Methods Enzymol.* 10 (1967) 245-250.
- [20] Yang Z.P., Dettbarn W.-D., Diisopropylphosphorofluoridate-induced cholinergic hyperactivity and lipid peroxidation, *Toxicol. Appl. Pharmacol.* 138 (1996) 48-53.
- [21] Yang Z.P., Morrow J.D., Wu A., Roberts L.J. II, Dettbarn W.-D., Diisopropylphosphorofluoridate-induced muscle hyperactivity associated with enhanced lipid peroxidation in vivo, *Biochem. Pharmacol.* 52 (1996) 357-361.

Regulation of gene expression by trans-synaptic activity: A role for the transcription factor NF- κ B

Victor Gisiger

*Département de Pathologie et Biologie cellulaire and Centre de Recherche en Sciences Neurologiques, Université de Montréal, C.P. 6128,
Succ. Centre-Ville, Montreal (Québec), Canada H3C 3J7*

Abstract — Earlier studies in the sympathetic ganglion have led to the proposal that adaptation of transcription to trans-synaptic activity is controlled by a signal transduction pathway featuring a transcription factor which translocates to the nucleus upon its release from the post-synaptic membrane by after-hyperpolarization. In light of recent progress, it is proposed here that NF- κ B constitutes the postulated transcription factor. (©Elsevier, Paris)

Résumé — **Régulation de la transcription par l'activité trans-synaptique: un rôle pour le facteur de transcription NF- κ B.** Des études antérieures ont amené à formuler l'hypothèse que, dans les neurones sympathiques, la transcription s'adapte à l'activité trans-synaptique grâce à un mécanisme de transduction par lequel un facteur de transcription, attaché à la membrane postsynaptique, est libéré par l'hyperpolarisation post-stimulatoire et migre dans le noyau. À la lumière de données nouvelles, il est proposé ici que NF- κ B constitue ce facteur de transcription. (©Elsevier, Paris)

NF- κ B / trans-synaptic regulation / gene expression / superior cervical ganglion

1. Introduction

It is now well established that activation of synaptic receptors by neurotransmitter can modify gene transcription and protein synthesis. However, the mechanisms whereby receptor activation sends transcriptional instructions from the post-synaptic membrane to the nucleus remain to be elucidated. One difficulty arises from the existence of several types of neurotransmitter-driven adaptations, each with its own mode of signal transduction. A textbook example is long-term potentiation (LTP), in which synaptic stimuli induce transcriptional changes through a second messenger signalling system, to eventually alter synaptic responsiveness [20, 22]. However, there is also a more basic and common type of neurotransmitter-driven adaptation whereby, as already hinted by Nissl more than a century ago [23], neurons adjust their protein content to their activity. Increasing data support the notion that all mature innervated excitable cells continuously adapt their protein composition and content to the amount of activity imposed upon them trans-synaptically [27, 31] (see references in [22, 29]). A dose-response relationship has been reported, for instance, between RNA synthesis and the amount (frequency and duration) of trans-synaptic stimulation in sympathetic ganglion [8, 12]. Altogether, the available data suggest that, in excitable cells, transcription of a number

of genes is constitutively locked on synaptic activity and continuously adjusts to its fluctuations.

This basic mode of adaptation to elicited activity has been extensively studied in the rat superior cervical ganglion (SCG), starting in the late sixties with the findings that trans-synaptic activation of sympathetic neurons increases the synthesis of RNA [13] and that of tyrosine hydroxylase (TH) [32]. These two independent lines of investigations yielded strikingly parallel results [3, 7, 12, 30, 33], suggesting that both RNA and TH adaptations are induced through the same regulatory mechanisms. The RNA studies allowed for the identification of the specific ionic event triggering the activity-mediated induction of RNA synthesis [9] and led to the proposal that sympathetic neurons adapt to activity through a direct synapse-to-nucleus signal transduction system involving the translocation of a protein able to modify gene transcription [8, 10–12].

More recently, reports about the transcription factor NF- κ B in the central nervous system (for review see [25]) have renewed interest in this proposal. Indeed, the properties displayed by NF- κ B in nervous tissue closely match those predicted for the regulatory protein in the postulated signal transduction system. Revisiting the earlier data in light of these recent developments improves the current understanding of the signal transduction pathway by which SCG adapts to trans-synaptic activation.

2. The regulation of RNA synthesis by neuronal activity

The regulation of RNA synthesis by trans-synaptic activation was investigated in incubated rat SCG by monitoring the changes in RNA radiolabelling brought about by preganglionic stimulation, or application of ACh or carbachol [8] ([12] and references cited therein). The results of these studies may be summarized as follows.

Prolonged stimulation of SCG induces a sequence of three changes in RNA synthesis: an initial decrease, followed by an increase developing after about 90 min of activation, and another and larger increase taking place after the end of the stimulation (see *figure 2* in [12]).

The three changes are specifically initiated by activation of the nicotinic receptors and the consecutive passive influx of Na^+ generating the fast EPSP. In particular, the effects of stimulation are abolished by replacing Na^+ of the medium by Tris or sucrose, or by clamping by KCl depolarization the membrane potential of the neurons near the reversal potential. Conversely, the RNA adaptations are independent of the generation of action potentials, membrane depolarization per se, activation of muscarinic receptors, as well as second messengers such as Ca^{2+} and cAMP.

Each of the three changes has been linked to a specific ionic event. The initial fall in RNA synthesis is caused by the Na^+ influx mediated by opening of the nicotinic receptors and, indeed, both elevation of internal Na^+ concentration ($[\text{Na}^+]_i$) [4] and decrease in RNA synthesis develop along parallel time-courses. Moreover, the initial ACh-mediated reduction in RNA synthesis can be reproduced without synaptic activation by application of aconitine, an alkaloid which, like batrachotoxin, prolongs the opening of fast Na^+ channels [2, 16]. Interestingly, the initial decrease in RNA synthesis affects all species of RNA and is accompanied by a broad, non-specific reduction in protein synthesis [14], suggesting that elevation of $[\text{Na}^+]_i$ depresses both transcription and translation machineries.

The increase in RNA synthesis occurring during the late stages of synaptic activation requires high external concentrations of Cl^- . Substitution of Cl^- by impermeant anions (sulfate or isethionate) abolishes this particular increase in RNA synthesis without affecting the two other ACh-mediated changes. As for the post-stimulation increase in RNA synthesis, it is related to the long-lasting after-hyperpolarization generated by the electrogenic extrusion of Na^+ by the $\text{Na}^+\text{-K}^+$ pump [5, 28]. There is a close parallelism between the development of both phenomena as well as the conditions which favour or

prevent their occurrence ([12] and references therein). Especially important, the post-stimulation increase in RNA synthesis is abolished by replacing NaCl in the incubating medium by LiCl , a condition which also prevents the after-hyperpolarization [5]. In contrast, the LiCl substitution does not affect the two changes, reduction and increase in RNA synthesis, occurring during the stimulation. In addition, an enhancement of RNA synthesis can be obtained without stimulation of the nicotinic receptors, by lowering the external KCl concentration or by applying tetrodotoxin to ganglia previously treated with aconitine, i.e., two conditions which generate an hyperpolarization in the sympathetic neurons [5] (references in [12]).

Both increases in RNA synthesis during and after activation, as monitored by 1-h radiolabelling, concern exclusively pre-messenger nuclear RNA [8, 10]. It was also demonstrated by autoradiography that these increases affect essentially sympathetic neurons, and not glial cells. Nevertheless, among the two increases in RNA synthesis, the one triggered by after-hyperpolarization is obviously that bearing the main functional significance. After-hyperpolarization appears to be a most appropriate signal for informing a regulation system of the level of activity to which to adapt. Indeed, the integral of amplitude and duration of the after-hyperpolarization is directly proportional to the amount of Na^+ extruded, which mirrors the influx of Na^+ [5]. Therefore, after-hyperpolarization provides the trans-synaptic regulation system with constantly up-dated information about the level of activity to which to adapt. This is especially relevant *in vivo* where the activation of sympathetic neurons occurs in a succession of irregularly spaced short trains of pulses [28].

Several facts, including the need for an adequate external Cl^- concentration to increase RNA synthesis during stimulation, make it unlikely that activation of the $\text{Na}^+\text{-K}^+$ pump or the reduction in $[\text{Na}^+]_i$ by themselves play a direct role in RNA regulation. The specific signal triggering the induction of RNA synthesis appears to be the hyperpolarization itself, i.e., the accumulation of negative charges in the sub-synaptic region. The regulation system controlling the adaptation to trans-synaptic activity should therefore be able to convert hyper-electronegativity into a signal bearing metabolic significance, and to transfer it to the nucleus within the lag of 15–30 min separating the generation of hyperpolarization and the actual onset of the RNA induction. For these reasons, a signal transduction pathway was postulated that comprises a transcription factor attached to the internal side of the post-synaptic membrane [8, 10, 12]. In this hypothesis, the accumulation of negative charges underneath the membrane generated by the

after-hyperpolarization would liberate this factor through an ion-exchange or electrostatic effect. The freed factor would then be channelled through the retrograde dendritic transport to the nucleus where it would associate with the promoter of the various genes subject to trans-synaptic regulation. The identity of this factor remained unknown. However, recent reports have revealed that the transcription factor NF- κ B displays properties in CNS neurons that match well those required by the model put forward [25]. Furthermore, rat SCG contains an inducible form of this factor (Gisiger and Bendayan, in preparation). Therefore, NF- κ B appears a likely candidate for the postulated factor transducing hyperpolarization into induction of RNA synthesis in rat SCG.

3. Role of the transcription factor NF- κ B in adaptation to activity

NF- κ B is an ubiquitous transcription factor specialized in generating rapid coordinated activation of genes in response to situations critical for survival (for reviews see [1, 25, 35]). Briefly, NF- κ B is stored in the cytoplasm, from where it rapidly translocates to the nucleus upon extracellular signaling, to enhance the transcription of numerous target genes. To accomplish this, NF- κ B carries two important sites: a nuclear localization signal (NLS), which directs the translocation to the nucleus, and a high affinity site toward a specific decameric sequence of DNA, the κ B site. Prior to the stimulus, NF- κ B remains sequestered in the cytoplasm in a latent form, because it is complexed to a specific inhibitory protein, I κ B, which covers these two sites. I κ B binds to the NLS of NF- κ B through an ankyrin-like region containing an acidic domain [6, 35]. Activation of NF- κ B occurs when, following an extracellular signal, I κ B is modified and dissociates from NF- κ B, allowing NF- κ B to translocate to the nucleus and to bind to the κ B DNA sequence.

Three recent observations are noteworthy in the present context. First, NF- κ B has been found associated with synaptic structures in adult cerebral cortex and hippocampus [15, 18]. Second, a 1–5 min stimulation of glutamate receptors in cultured cerebellar granule cells induces translocation of NF- κ B to the nucleus of these neurons within a 30–45 min period, but not in glial cells [15, 19]. Third, it has been shown in *Aplysia* that soluble cytoplasmic proteins containing the NLS are taken up by the retrograde axonal transport, probably through a receptor, and rapidly carried into the nucleus [26].

These facts fit well into the proposed model of synapse-to-nucleus signal transduction. Neverthe-

less, further implications need to be considered. In order for NF- κ B to remain close to the synapses, as already described [15, 18, 19], there is a need for a synaptic variant of I κ B anchored to synaptic structures such as postsynaptic densities, for example [29]. In this instance, hyperpolarization, by counteracting the negative charges of the acidic domain of the I κ B ankyrin regions, would release NF- κ B at least as long as hyper-electronegativity persists. Having its NLS exposed, NF- κ B would then associate with the NLS receptors to undergo retrograde dendritic transport and translocation into the nucleus. Because of its ability to carry NF- κ B away from synaptic I κ B before hyperpolarization disappears, the proposed mechanism provides an efficient way to transform an ephemeral event, lasting no longer than a few seconds, into middle or long-term alterations of transcription.

Interestingly, a κ B-like motif is present within the promoter of TH [17, 21]. This suggests that the transcription of TH is directly enhanced by NF- κ B without the intermediary of other early genes. This is consistent with the observation that the AP-1 transcription factor does not mediate trans-synaptic induction of TH transcription in adult SCG [34]. Indirect evidence suggests that other genes, including choline-acetyl-transferase [24] and dopamine- β -hydroxylase ([33] and references therein), have their transcription controlled by the same synapse-to-nucleus signalling system. The promoter of these genes should contain the specific κ B DNA sequence, which will be helpful for their identification. Taking into account the recently reported localization of NF- κ B at central synapses, it is possible that a similar synapse-to-nucleus signal transduction pathway accounts for adaptations to trans-synaptic activation in the CNS.

Acknowledgments

I thank Drs. Laurent Descarries and Phillip Gardiner for their interest and critical reading of the manuscript.

References

- [1] Baldwin A.S. Jr, The NF- κ B and I κ B proteins: new discoveries and insights, *Annu. Rev. Immunol.* 14 (1996) 649–681.
- [2] Ballanyi K., Grafe P., Reddy M.M., Ten Bruggencate G., Different types of potassium transport linked to carbachol and γ -aminobutyric acid actions in sympathetic neurons, *Neuroscience* 12 (1984) 917–927.
- [3] Bönnisch H., Otten U., Thoenen H., The role of sodium influx mediated by nicotinic receptors as an initial event in trans-synaptic induction of tyrosine hydroxylase in adrenergic neu-

- rons, Naunyn-Schmiedeberg's Arch. Pharmacol. 313 (1980) 199-203.
- [4] Brown D.A., Scholfield C.N., Changes of intracellular sodium and potassium ion concentrations in isolated rat superior cervical ganglia induced by depolarizing agents, *J. Physiol. (Lond.)* 242 (1974) 307-319.
 - [5] Brown D.A., Brownstein M.J., Scholfield C.N., Origin of the after-hyperpolarization that follows removal of depolarizing agents from the isolated superior cervical ganglion of the rat, *Br. J. Pharmacol.* 44 (1972) 651-671.
 - [6] Ernst M.K., Dunn L.L., Rice N.R., The PEST-like sequence of I κ B α is responsible for inhibition of DNA binding but not for cytoplasmic retention of c-Rel or RelA homodimers, *Mol. Cell. Biol.* 15 (1995) 872-882.
 - [7] Faucon Biguet N., Rittenhouse A.R., Mallet J., Zigmond R.E., Preganglionic nerve stimulation increases mRNA levels for tyrosine hydroxylase in rat superior cervical ganglion, *Neurosci. Lett.* 104 (1989) 189-194.
 - [8] Gisiger V., Triggering of RNA synthesis by acetylcholine stimulation of the postsynaptic membrane in a mammalian sympathetic ganglion, *Brain Res.* 33 (1971) 139-146.
 - [9] Gisiger V., Rôles des ions Na⁺, K⁺ and Cl⁻ et de l'hyperpolarisation dans la régulation de la synthèse de l'ARN des neurones sympathiques, *C.R. Acad. Sci. Paris, Série D*, 277 (1973) 233-235.
 - [10] Gisiger V., Ionic contribution to the regulation of RNA metabolism in the neurones, in: Matthies H. (Ed.), *Proc IVth Internat Neurobiol Symp Magdeburg 1973*, V.E.B. Verlag, Berlin, 1974, pp. 236-246.
 - [11] Gisiger V., The regulation of RNA and protein metabolism by neuronal activity: recent results with peripheral nervous tissue, in: Frigyesi T.L. (Ed.), *Subcortical Mechanisms and Sensorimotor Activities*, Hans Huber, Bern, 1975, pp. 47-61.
 - [12] Gisiger V., Role of hyperpolarization generated by the Na⁺-K⁺ pump in the trans-synaptic induction of RNA synthesis in sympathetic neurons, 12th Gif Lectures in Neurobiology, *J. Physiol. (Paris)* 83 (1988-89) 148-163.
 - [13] Gisiger V., Gaide-Huguenin A.-C., Effect of preganglionic stimulation upon RNA synthesis in the isolated sympathetic ganglion of the rat, *Progr. Brain Res.* 31 (1969) 125-129.
 - [14] Gisiger V., Venkov L., Gautron J., Acetylcholinesterase and biosynthesis of proteins in the resting and stimulated sympathetic ganglion, *J. Neurochem.* 25 (1975) 737-748.
 - [15] Guerrini L., Blasi F., Denis-Donini S., Synaptic activation of NF- κ B by glutamate in cerebellar granule neurons in vitro, *Proc. Natl. Acad. Sci. USA* 92 (1995) 9077-9081.
 - [16] Hille B., *Ionic channels of excitable membranes*, Sinauer Associates Inc., Sunderland, Massachusetts, 1984.
 - [17] Icard-Liepkalns C., Faucon Biguet N., Vyas S., Robert J.J., Sassone-Corsi P., Mallet J., AP-1 complex and c-fos transcription are involved in TPA provoked and trans-synaptic inductions of the tyrosine hydroxylase gene: insights into long-term regulatory mechanisms, *J. Neurosci. Res.* 32 (1992) 290-298.
 - [18] Kaltschmidt C., Kaltschmidt B., Bauerle P.A., Brain synapses contain inducible forms of the transcription factor NF- κ B, *Mech. Dev.* 43 (1993) 135-147.
 - [19] Kaltschmidt C., Kaltschmidt B., Bauerle P.A., Stimulation of ionotropic glutamate receptors activates transcription factor NF- κ B in primary neurons, *Proc. Natl. Acad. Sci. USA* 92 (1995) 9618-9622.
 - [20] Kandel E.R., Schwartz J.H., Jessell T.M., *Principles of neural science*, Appleton & Lange, Norwalk, Connecticut, 1991.
 - [21] Kumer S.C., Vrana K.E., Intricate regulation of tyrosine hydroxylase activity and gene expression, *J. Neurochem.* 67 (1996) 443-462.
 - [22] Mackler S.A., Brooks B.P., Eberwine J.H., Stimulus-induced coordinate changes in mRNA abundance in single postsynaptic hippocampal CA1 neurons, *Neuron* 9 (1992) 539-548.
 - [23] Nissl F., Über die Veränderungen der Ganglienzellen am Facialis des Kaninchens nach Ausreissung des Nerven, *Allg. Z. Psychiat.* 48 (1892) 197-198.
 - [24] Oesch F., Thoenen H., Increased activity of the peripheral sympathetic nervous system: induction of choline acetyltransferase in the preganglionic cholinergic neurone, *Nature* 242 (1973) 536-537.
 - [25] O'Neill L.A.J., Kaltschmidt C., NF- κ B: a crucial transcription factor for glial and neuronal cell function, *Trends Neurosci.* 20 (1997) 252-258.
 - [26] Schmied R., Huang C.-C., Zhang X.-P., Ambron D.A., Ambron R.T., Endogenous axoplasmic proteins and proteins containing nuclear localization signal sequences use the retrograde axonal transport/nuclear import pathway in *Aplysia* neurons, *J. Neurosci.* 13 (1993) 4064-4071.
 - [27] Sheng M., Greenberg M.E., The regulation and function of c-fos and other immediate early genes in the nervous system, *Neuron* 4 (1990) 477-485.
 - [28] Skok V.I., Ganglionic transmission: morphology and physiology, in: Kharkevich D.A. (Ed.), *Pharmacology of ganglionic transmission*, Springer-Verlag, Berlin, 1980, pp. 9-39.
 - [29] Suzuki T., Mitake S., Okumura-Noji K., Yang J.-P., Fujii T., Okamoto T., Presence of NF- κ B-like and I κ B-like immunoreactivities in postsynaptic densities, *NeuroReport* 8 (1997) 2931-2935.
 - [30] Thoenen H., Otten U., Trans-synaptic enzyme induction: ionic requirements and modulatory role of glucocorticoids, in: Usdin E., Weiner N., Youdim M.B.H. (Eds.), *Structure and function of monoamine enzymes*, M. Dekker Inc., New York, 1978, pp. 439-464.
 - [31] Thoenen H., Edgar D., The regulation of neuronal gene expression, *Trends Neurosci.* 5 (1982) 311-313.
 - [32] Thoenen H., Mueller R.A., Axelrod J., Increased tyrosine hydroxylase activity after drug-induced alteration of sympathetic transmission, *Nature* 221 (1969) 1264.
 - [33] Thoenen H., Otten U., Schwab M., Orthograde and retrograde signals for the regulation of neuronal gene expression: the peripheral sympathetic nervous system as a model, in: Schmitt F.O., Worden F.G. (Eds.), *The Neurosciences, Fourth study program*, MIT Press, Cambridge, 1979, pp. 911-928.
 - [34] Trocmé C., Mallet J., Faucon Biguet N., AP-1 mediates trans-synaptic induction of tyrosine hydroxylase gene expression in adrenal medulla but not in superior cervical ganglia, *J. Neurosci. Res.* 48 (1997) 489-498.
 - [35] Verma I.M., Stebensson J.K., Schwarz E.M., Van Antwerp D., Miyamoto S., Rel/NF- κ B/I κ B family: intimate tales of association and dissociation, *Genes Dev.* 9 (1995) 2723-2735.

Development of the neuromuscular junction: Genetic analysis in mice

Joshua R. Sanes^a, Elizabeth D. Apela^a, Robert W. Burgess^a, Ronald B. Emerson^b,
Guoping Feng^a, Medha Gautam^{a*}, David Glass^d, R. Mark Grady^a, Eric Krejci^c,
Jeff W. Lichtman^a, Jonathan T. Lu^b, Jean Massoulié^c, Jeffrey H. Miner^{a*}, Lisa M. Moscoso^a,
Quyen Nguyen^a, Mia Nichol^a, Peter G. Noakes^{a*}, Bruce L. Patton^a, Young-Jin Son^{a*},
George D. Yancopoulos^d, Heather Zhou^a

^aDepartment of Anatomy, Washington University School of Medicine, 660 S. Euclid Avenue, Campus Box 8108, St. Louis, MO 63110, USA

^bDepartment of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN, USA

^cLaboratoire de Neurobiologie Cellulaire et Moléculaire, École Normale Supérieure, Paris, France

^dRegeneron Pharmaceuticals, Tarrytown, NY, USA

Abstract — Formation of the skeletal neuromuscular junction is a multi-step process that requires communication between the nerve and muscle. Studies in many laboratories have led to identification of factors that seem likely to mediate these interactions. ‘Knock-out’ mice have now been generated with mutations in several genes that encode candidate transsynaptic messengers and components of their effector mechanisms. Using these mice, it is possible to test hypotheses about the control of synaptogenesis. Here, we review our studies on neuromuscular development in mutant mice lacking agrin α CGRP, rapsyn, MuSK, dystrophin, dystrobrevin, utrophin, laminin α 5, laminin β 2, collagen α 3 (IV), the acetylcholine receptor ϵ subunit, the collagenous tail of acetylcholinesterase, fibroblast growth factor-5, the neural cell adhesion molecule, and tenascin-C. (©Elsevier, Paris)

Résumé — Développement de la jonction neuromusculaire : analyse génétique chez la souris. La formation de la jonction neuromusculaire des muscles squelettiques est un processus à étapes multiples qui requiert une communication entre nerf et muscle. Les études de nombreux laboratoires ont conduit à l’identification de facteurs qui semblent capables d’assurer ces interactions. Chez la souris, nous avons inactivé (‘knock out’) les gènes de plusieurs messagers trans-synaptiques potentiels et de composants de leurs mécanismes effecteurs. Grâce à ces souris, il est possible d’analyser le contrôle de la synaptogénèse. Nous décrivons ici les résultats obtenus chez les souris mutantes dépourvues des protéines suivantes : agrine, α CGRP, rapsyne, MuSK, dystrophine, dystrobrevine, utrophine, laminine α 5, laminine β 2, collagène α 3 (IV), sous-unité ϵ du récepteur nicotinique, queue collagénique de l’acétylcholinestérase, FGF-5, N-CAM et ténascine-C. (©Elsevier, Paris)

acetylcholine receptor / agrin / basal lamina / laminin / synapse formation

1. Introduction

The vertebrate skeletal neuromuscular junction (NMJ) is the best understood of all synapses. Its accessibility and simplicity led to its being studied intensively over most of this century, so we now know an enormous amount about its structure, function and molecular architecture. For example, numerous proteins have been identified as components of the pre- and postsynaptic membranes, the synaptic vesicles that fill the nerve terminal, the Schwann cell that caps the terminal, the cytoskeleton that coats the infolded postsynaptic membrane, and the basal lamina that runs through the synaptic cleft (*figure 1*).

This rich background, in turn, has facilitated developmental analysis of the NMJ. Studies carried out

over the past 30 years have shown that the NMJ develops in a series of steps that involve and require complex interactions between nerve and muscle. That is, the growth cone of a motor axon initiates formation of the postsynaptic apparatus, the muscle cell in turn provides retrograde signals that organize the transformation of a growth cone into a nerve terminal, the nerve terminal promotes postsynaptic maturation, and so on. Attempts to elucidate the mechanisms underlying these interactions have led to identification of numerous molecules that may serve as intercellular signals or components of their intracellular signal transduction pathways. Many of these molecules have bioactivities *in vitro* and localizations *in vivo* consistent with their involvement in synaptic development. It has been difficult, however, to determine what roles, if any, these candidates play *in vivo*.

Recently, the ‘knockout’ technique has provided a way to circumvent this limitation in mice. Mutations can be engineered in genes by homologous recombination in embryonic stem (ES) cells. The ES

* Present addresses: M. Gautam, Department of Pharmacology, St. Louis University School of Medicine, St. Louis, MO, USA; J. Miner, Department of Medicine, Washington University, USA; P. Noakes, University of Queensland, Brisbane, Australia; Young-Jin Son, Department of Physiology, University of Washington, Seattle, WA, USA.

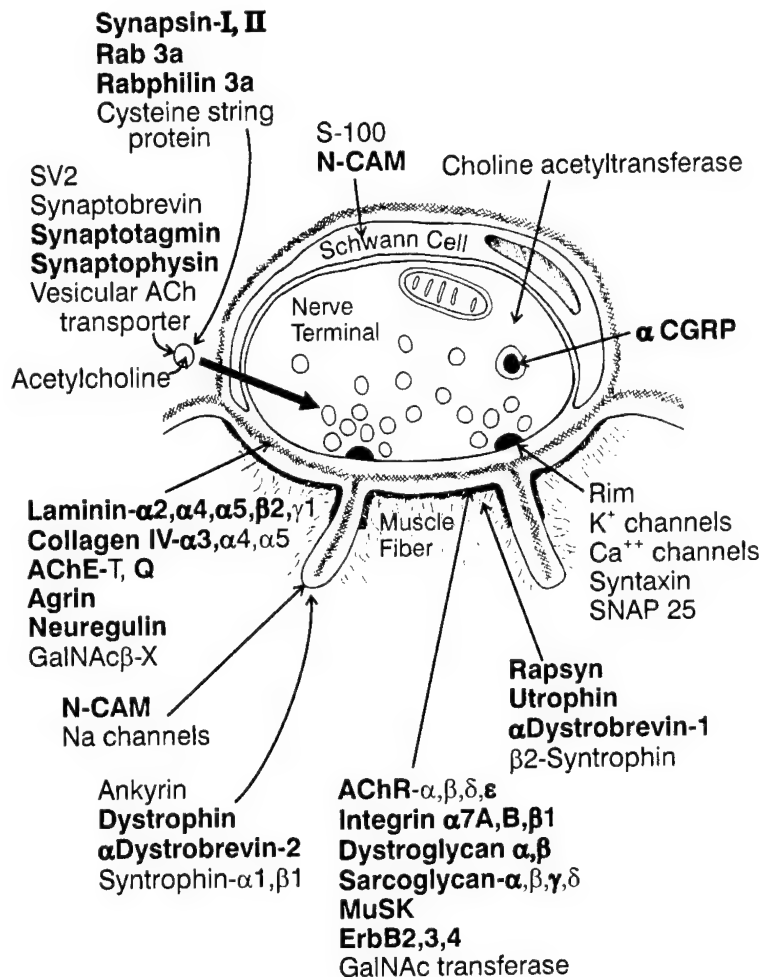


Figure 1. Structure and molecular architecture of the NMJ. The nerve terminal occupies a shallow gutter in the muscle fiber and is capped by processes of Schwann cells. Active zones in the nerve terminal directly appose junctional folds in the postsynaptic membrane. Some of the proteins concentrated at the synapse are shown, with their subcellular localizations indicated by arrows. Those for which 'knock-out' mice have been generated are indicated in bold-face. See Hall and Sanes [15] and Sanes and Lichtman [25] for primary references and names of additional components.

cells are then injected into the blastocyst to generate, in sequence, germ-line chimeras, mutant heterozygotes (which usually exhibit no detectable phenotype) and homozygotes (which sometimes do). In work begun in collaboration with the late John Merlie, our group has generated and/or analyzed several of these mutants. This chapter summarizes some of these studies. Limitations of space preclude citing the studies that led to our work; we refer the reader to reviews by Hall and Sanes [15], Duclert and Changeux [6], Bowe and Fallon [2], Fischbach and Rosen [8], Daniels [4], and Sanes and Lichtman [25].

2. Agrin: a hypomorph, a null, and an isoform-specific mutant

Nerves and components of synaptic basal lamina can induce clustering of acetylcholine receptors

(AChRs) in the postsynaptic membrane of the NMJ. Several groups therefore sought factors that could stimulate the clustering of initially diffuse AChRs on aneural cultured myotubes. Using this assay, McMahan and colleagues isolated a protein that they called agrin. They then generated antibodies, and used them to show that agrin is synthesized by motoneurons, transported down motor axons, and stably incorporated into the basal lamina of the synaptic cleft. Based on these studies, McMahan proposed that agrin is a critical nerve-derived organizer of postsynaptic differentiation [2].

We generated agrin-deficient mutant mice to test this hypothesis. The original mutants we analyzed were severe hypomorphs, that expressed no 'active' agrin (see below) at all, and only ~10% of wild-type levels of other agrin isoforms [9]. More recently, we have generated a protein null and obtained similar results [3]. In both the hypomorph and the null, post-

synaptic differentiation is drastically impaired: neither clustered AChR clusters nor other synaptic specializations are detectable at most motor axon-myotube contacts. Those AChR clusters that are present are smaller and less densely packed than normal, and most often occur on uninervated portions of the myotube surface. These results support McMahan's 'agrin hypothesis'. In addition, mutant motor nerve terminals fail to arborize or differentiate normally.

Although agrin is confined to synaptic sites in adult muscle fibers, it is synthesized by muscle as well as nerve and is present throughout the basal lamina of developing myotubes. Initially, this distribution seemed inconsistent with the hypothesis that agrin serves as a nerve-derived signal. A likely resolution to this paradox came with the discoveries that an alternatively spliced product of the agrin gene (which includes exons called 'B' in birds and 'z' in mammals) greatly potentiates the AChR clustering activity of agrin, and is expressed only by neurons. Our initial analysis did not distinguish between roles of neural and muscle agrin. We addressed this issue in two ways [3]. First, agrin^{-/-} muscles were transplanted to wild-type hosts, where they were reinnervated by agrin-expressing axons. Synapses in these surgical chimeras displayed neither pre- nor postsynaptic defects. Second, a novel allele was generated in which the 'z' exons were deleted with only minor effects on the levels of 'z-minus' muscle agrin. Both pre- and postsynaptic defects in these 'second generation' mutants were indistinguishable from those in the original allele. Thus, it is nerve-derived z agrin that is essential for synaptic development. Moreover, the effects of agrin on motor axons are not mediated by muscle agrin, but are more likely to be an indirect consequence of impaired postsynaptic differentiation.

3. Agrin signalling: rapsyn and MuSK

Rapsyn is a 43 kDa cytoplasmic protein originally isolated by virtue of co-purification with AChRs. It is precisely co-localized with AChRs at the NMJ, and causes redistribution of diffusely distributed AChRs into high-density patches when co-expressed with them in heterologous cells. These results suggested an involvement of rapsyn in AChR clustering at the synapse. Genetic analysis supported this idea: AChRs were synthesized but failed to cluster in muscles of rapsyn^{-/-} mice or in myotubes cultured from the mutants, even in the presence of high levels of agrin [10]. Likewise, numerous components of the postsynaptic membrane and cytoskeleton remained diffusely distributed in mutants [19]. Thus, rapsyn is necessary for agrin to exert its effects.

How does agrin influence rapsyn? Agrin interacts with numerous components of the myotube surface, several of which seemed reasonable candidates to mediate agrin's effects on postsynaptic differentiation [24]. However, mutations that disrupt some of them fail to show severe neuromuscular defects ([18] and see below). In contrast, the phenotype of 'knock-out' mice lacking a muscle-specific tyrosine kinase (MuSK) exhibited a phenotype similar to, and if anything, more severe than that of the agrin mutants described above [5] (Gautam et al., submitted). Based on this phenotype, MuSK, which had been isolated as part of an unrelated study, became the leading candidate agrin receptor. In support of this idea, MuSK^{-/-} myotubes are unresponsive to agrin, responsiveness is restored by reintroduction of MuSK, and a dominant negative MuSK protein inhibits agrin signaling in wild-type muscle cells [11, 12] (Zhou and Sanes, in preparation).

Although genetic studies place agrin, MuSK and rapsyn in a common pathway, they leave open questions of what steps intervene between agrin and MuSK and between MuSK and rapsyn. Isolated MuSK cannot bind agrin, so it is likely to be only one component of a multi-subunit receptor. Other subunits (called MASCs for muscle-associated specificity components; [12]) have not yet been identified. Likewise, the interaction of MuSK with rapsyn is complex: although MuSK and rapsyn co-cluster in heterologous cells, it is the extracellular rather than the cytoplasmic domain of MuSK that is essential for clustering. Because rapsyn is entirely cytoplasmic, this result implies the existence of a rapsyn-associated transmembrane linker (RATL; [1]). Rapsyn does not appear to localize MuSK to synapses, however, in that MuSK is clustered at synaptic sites in rapsyn^{-/-} mice [1]. Thus, MuSK is a critical component of a primary synaptic scaffold and rapsyn recruits other synaptic components to that scaffold. A final complexity is that rapsyn and AChRs cluster spontaneously in heterologous cells, but fail to cluster in myotubes unless agrin (or another exogenous agent is added). The implication is that muscles normally exert some inhibitory influence on clustering, which agrin relieves via MuSK.

4. The dystrophin-glycoprotein complex: utrophin, dystrophin, and α -dystrobrevin

Dystroglycan was isolated as part of a multimolecular complex (the dystrophin glycoprotein complex or DGC) that co-purifies with dystrophin, the cytoskeletal protein mutated in humans with Duchenne or Becker muscular dystrophy and in mdx mice. Dystroglycan binds to laminin extracellularly

and dystrophin intracellularly, forming part of a system that links the extracellular matrix to the cytoskeleton. The DGC is also present at the NMJ, but with alterations, the most striking of which is that dystrophin is replaced by an autosomal homologue, utrophin at the synapse.

In 1994, several groups reported that agrin binds tightly and specifically to dystroglycan, leading to the speculation that dystroglycan might be the functional agrin receptor, and to the specific hypothesis that utrophin transforms a widely distributed laminin receptor into an agrin receptor. Dystroglycan^{-/-} mutants die before muscles form, and are therefore unsuitable for studies of the NMJ. However, it was possible to test the specific hypothesis that utrophin is essential for synaptic development. In fact, utrophin^{-/-} mice are viable, fertile, and apparently healthy. Overall, their NMJs are qualitatively normal, but the density of AChRs in the postsynaptic membrane is reduced by ~30% in mutants compared to controls, and the number of junctional folds is reduced by half [13]. One possible explanation for the subtlety of the defects in utrophin^{-/-} mice was that the presence of dystrophin provided compensation for loss of its homologue. We tested this possibility by generating and analyzing double mutants lacking both utrophin and dystrophin. The double mutants were severely dystrophic, but their NMJs were not significantly more affected than those of utrophin^{-/-} or dystrophin^{-/-} mice [14]. Thus, utrophin and dystrophin are dispensable for formation of the NMJ.

More recently, we generated mice lacking another cytoplasmic component of the DGC, α -dystrobrevin. These mice exhibit a mild dystrophy similar to that seen in dystrophin^{-/-} mice. Interestingly, however, AChR-rich regions at α -dystrobrevin^{-/-} NMJs have a feathered appearance not seen in utrophin^{-/-}, dystrophin^{-/-} or double mutant synapses. Moreover, agrin induces significantly smaller AChR clusters in α -dystrobrevin^{-/-} than in wild-type myotubes [26]. These results raise the possibility that the DGC may be involved in the maturation or maintenance of the NMJ, perhaps by stabilizing postsynaptic specializations once they have been generated through a MuSK-dependent pathway.

5. AChR synthesis: neuregulins and α CGRP

Motor nerves not only cluster diffusely-distributed AChRs but also induce selective transcription of AChR genes by subsynaptic nuclei. Three molecules have been proposed as nerve-derived inducers of AChR gene expression: neuregulin, α CGRP, and agrin itself. All three are reasonable candidates based on their localizations and bioactivities. Evidence in

favor of neuregulin and agrin is reviewed elsewhere [8, 25]. α CGRP is a peptide generated from an alternatively spliced product of the gene that also encodes calcitonin. α CGRP is synthesized by motoneurons, released by motor nerve terminals, and capable of stimulating AChR expression by cultured myotubes. α CGRP has also been proposed to regulate activity-dependent sprouting of motor axons. However, we detected no defects in synaptic development or regeneration in mutant mice lacking α CGRP (Lu et al., in preparation). In particular, synaptic nuclei became transcriptionally specialized in the mutants. One potential caveat to this finding is that the closely related peptide β CGRP might have compensated for lack of its homologue. However, antisera that recognize both α and β CGRP stained motor nerve terminals in wild-type but not in α CGRP^{-/-} mice, implying that neither CGRP is required for synaptogenesis.

6. Synaptic basal lamina: laminins α 5 and β 2 and collagen α 3 (IV)

Studies on regeneration on nerve and muscle revealed that synaptic basal lamina contains components capable of organizing postsynaptic differentiation in the absence of the nerve and presynaptic differentiation in the absence of muscle. In addition, components of basal lamina account, at least in part, for the preferential reinnervation of original synaptic sites following axotomy [15]. Apparently, nerve terminals and muscle fibers insert components into synaptic basal lamina to influence each other's differentiation or regeneration. Agrin, which is inserted by the nerve, is clearly a major organizer of postsynaptic differentiation. Likely candidates for muscle-derived retrograde signals are synapse-specific isoforms of widely-distributed basal lamina components. The entire muscle fiber basal lamina is rich in laminin and collagen IV, but isoforms differ between synaptic and extrasynaptic regions. Specifically, extrasynaptic laminin is rich in laminin 2 (composed of the β 2, β 1 and γ 1 subunits) whereas laminin 11 (α 5/ β 2/ γ 1) is one of a few major synaptic laminins. Similarly, extrasynaptic and synaptic basal lamina are rich in the α 1/ β 2 and α 3- α 5 chains of collagen IV, respectively. Moreover, native laminin 11 and the isolated β 2 chain serve as stop signals for growing motor neurites, and β 2 fragments trigger differentiation of growth cones into motor nerve terminals [23].

To test roles of laminins and collagens in synaptic development, we generated mutants lacking laminin α 5, laminin β 2, and collagen α 3 (IV). Laminin α 5 mutants die of placental defects just as NMJs are

beginning to form, and collagen $\alpha 3$ (IV) show no obvious neuromuscular defects, but laminin $\beta 2$ mutants have been informative. Formation of nerve terminals is severely impaired in laminin $\beta 2^{-/-}$ mice: vesicles fail to aggregate near the presynaptic membrane, few active zones form, and levels of spontaneous and evoked transmitter release are decreased several-fold [20]. The consequent weakness is a major contributor, along with renal defects [21] to failure of the mutants to thrive and to their death during the third postnatal week.

Synaptic laminins also provide an inhibitory signal to the terminal Schwann cell. In normal muscle, Schwann cells cap nerve terminals, but in laminin $\beta 2^{-/-}$ mutants, Schwann cell processes invade the synaptic cleft. Although we initially speculated that this defect was a secondary consequence of decreased adhesion of nerve to basal lamina, experiments *in vivo* and *in vitro* showed that synaptic laminins actively inhibit extension of Schwann cell processes [22]. Thus, maintenance of normal nerve-muscle apposition requires not only tight adhesion of nerve to muscle via the intervening cleft, but active repulsion of the Schwann cell from the cleft. This interaction, in turn, may be necessary because Schwann cells need to be in close apposition to nerve terminals to provide them with with trophic sustenance. Repulsion from the cleft allows the nerve terminal-Schwann cell relationship to be intimate without becoming suffocating.

7. Retrograde messengers: N-CAM, FGF-5, and tenascin-C

Several proteins originally shown to be neuroactive in other systems were later implicated in the control of neuromuscular development. For example, the neural cell adhesion molecule (N-CAM) is co-regulated with AChRs during development and following denervation, binds agrin tightly and specifically, and promotes neurite outgrowth. Moreover, infusion of antibodies to N-CAM into denervated muscle interferes with reinnervation. Fibroblast growth factor 5 (FGF-5) is a potent promoter of motoneuron survival and differentiation *in vitro*, and accounts for a large fraction of the survival-promoting activity extractable from neonatal skeletal muscle. Tenascin C is synthesized by a population of perisynaptic fibroblasts that is stimulated to proliferate following denervation; this intriguing distribution along with antibody-blocking experiments suggested that tenascin-C might help guide regenerating axons back to original synaptic sites (see [18] for reference).

We tested these hypotheses by analyzing mutant mice lacking N-CAM, FGF-5, tenascin-C, or both N-CAM and tenascin-C. In brief, synaptic development, reinnervation, and sprouting following partial denervation proceeded normally in all three mutants. Two aspects of postnatal synaptic maturation were slowed in the N-CAM $^{-/-}$ mutants: the generation of junction folds and the elimination of polyneuronal innervation. Nonetheless, all three molecules are dispensable for most aspects of synaptic development.

8. Machinery of neurotransmission: AChRs and acetylcholinesterase

The point of neuromuscular development is to form the specializations required for neurotransmission. Prominent among these are AChRs and acetylcholinesterase (AChE). In addition to their roles in synaptic function, however, both of these proteins have been hypothesized to play roles in synaptic development. We used genetic methods to test this idea.

The subunit composition of AChRs changes postnatally: AChRs containing a gamma subunit ($\alpha 2\beta\gamma\delta$) are replaced by AChRs containing a homologous epsilon subunit ($\alpha 2\beta\epsilon\delta$) during the first postnatal week. The significance of the switch may be in biophysical differences between the subunits: ϵ -containing AChRs open more briefly but with higher conductance than γ -AChRs. Another possibility, though, is that the transition is important for structural maturation of the synapse. For example, the γ and ϵ subunits might interact with different proteins. We tested this idea in ϵ -deficient mutants. Levels of AChRs are initially normal in these mutants, and γ -AChRs are retained long beyond the time they normally disappear. Even while the γ subunit is providing compensation, the formation of the raised end-plate and of junctional folds are impaired. Later, as AChR levels decline, other synaptic components are lost in parallel, and the remaining components are collected into micro-islands [17]. Together, these results reveal novel structural roles of the AChR.

Much of the AChE of the NMJ is anchored to the synaptic basal lamina via a collagenous subunit called 'Q.' We generated Q-deficient mutants, and showed that they lacked all detectable synaptic AChE. Homozygous mutants were weak and survival was variable, but some mutants lived for several months. In view of several reports suggesting that AChE modulates neurite outgrowth, we were particularly interested in the possibility that motor axons might fail to stop growing or to arborize properly at AChE-deficient end-plates. In fact, arborization

was grossly normal in the mutants, but the precise apposition of pre- to postsynaptic specializations was degraded. Ongoing work is aimed at determining whether this phenotype reflects a signaling function of AChE or whether it is secondary to altered activity patterns.

9. Conclusion

Analysis of the mutant phenotypes is by no means straightforward, in that significant roles can be masked by redundancy or compensation, and apparently significant defects can result from indirect effects. Nonetheless, mutant mice have allowed some tests of hypothesis about mechanisms that regulate neuromuscular development. For example, they have provided evidence for a pathway of postsynaptic differentiation in which agrin is a nerve-derived signal, MuSK is a component of the receptor, rapsyn is an effector, and the DGC is involved in stabilization. Genetic studies have also allowed tests of candidate retrograde signals, and implicated the synaptic laminins in this process. An intriguing question for the future, which the mutants may help us answer, is whether similar mechanisms regulate formation of neuron-neuron synapses in ganglia or the brain [7].

References

- [1] Apel E.D., Glass D.J., Moscoso L.M., Yancopoulos G.D., Sanes J.R., Rapsyn is required for MuSK signaling and recruits synaptic components a MuSK-containing scaffold. *Neuron* 18 (1997) 623–635.
- [2] Bowe M.A., Fallon J.R., The role of agrin in synapse formation. *Annu. Rev. Neurosci.* 18 (1995) 443–462.
- [3] Burgess R.W., Nguyen Q., Lichtman J.W., Sanes J.R., A genetic analysis of agrin. *Neurosci. Abstr.* (1998), in press.
- [4] Daniels M.P., Intercellular communication that mediates formation of the neuromuscular junction. *Mol. Neurobiol.* 14 (1997) 143–170.
- [5] DeChiara T.M., Bowen D.C., Valenzuela D.M., Simmons M.V., Poueymirou W.T., Thomas S., Kinetz E., Compton D.L., Rojas E., Park J.S., Smith C., DiStefano P.S., Glass D.J., Burden S.J., Yancopoulos G.D., The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell* 85 (1996) 501–512.
- [6] Duclert A., Changeux J.P., Acetylcholine receptor gene expression at the developing neuromuscular junction. *Physiol. Rev.* 75 (1995) 339–368.
- [7] Feng G., Steinbach J.H., Sanes J.R., Rapsyn clusters neuronal acetylcholine receptors but is inessential for formation of an interneuronal cholinergic synapse. *J. Neurosci.* 18 (1998) 4166–4176.
- [8] Fischbach G.D., Rosen K.M., ARIA: A neuromuscular junction neuregulin. *Annu. Rev. Neurosci.* (1997) 429–458.
- [9] Gautam M., Noakes P.G., Moscoso L., Rupp F., Scheller R.H., Merlie J.P., Sanes J.R., Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* 85 (1996) 525–535.
- [10] Gautam M., Noakes P.G., Mudd J., Nichol M., Chu G.C., Sanes J.R., Merlie J.P., Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature* 37 (1995) 232–236.
- [11] Glass D.J., Apel E.D., Shah S., Bowen D.C., DeChiara T.M., Stitt T.N., Sanes J.R., Yancopoulos G.D., MuSK kinase domain sufficient for phosphorylation but not clustering of acetylcholine receptors. *Proc. Natl. Acad. Sci. USA* 94 (1997) 8848–8853.
- [12] Glass D.J., Bowen D.C., Stitt T.N., Radziejewski C., Bruno J.T., Gies D.R., Shah S., Mattsson K., Burden S.J., DiStefano P.S., Valenzuela D.M., DeChiara T.M., Yancopoulos G.D., Agrin acts via a MuSK receptor complex. *Cell* 85 (1996) 513–523.
- [13] Grady R.M., Merlie J.P., Sanes J.R., Subtle neuromuscular defects in utrophin-deficient mice. *J. Cell Biol.* 13 (1997) 871–882.
- [14] Grady R.M., Teng H., Nichol M.C., Cunningham J.C., Wilkinson R.S., Sanes J.R., Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. *Cell* 90 (1997) 729–738.
- [15] Hall Z.W., Sanes J.R., Synaptic structure and development: the neuromuscular junction. *Cell* 72 (1993) 99–121.
- [16] Miner J.H., Sanes J.R., Molecular and functional defects in kidneys of mice lacking collagen (α 3) IV: implications for alport syndrome. *J. Cell Biol.* 135 (1998) 1403–1413.
- [17] Missias A.C., Mudd J., Cunningham J.M., Steinbach J.H., Merlie J.P., Sanes J.R., Deficient development and maintenance of postsynaptic specializations in mutant mice lacking an 'adult' acetylcholine receptor subunit. *Development* 124 (1997) 5075–5086.
- [18] Moscoso L.M., Cremer H., Sanes J.R., Formation and regeneration of neuromuscular junctions in mice lacking N-CAM, tenascin-C, or FGF-5. *J. Neurosci.* 18 (1998) 1465–1477.
- [19] Moscoso L.M., Merlie J.P., Sanes J.R., N-CAM, 43k-rapsyn, and S-laminin mRNAs are concentrated at synaptic sites in muscle fibers. *Mol. Cell Neurosci.* 6 (1995) 80–89.
- [20] Noakes P.G., Gautam M., Mudd J., Sanes J.R., Merlie J.P., Aberrant differentiation of neuromuscular junctions in mice lacking s-laminin/laminin β 2. *Nature* 374 (1995) 258–262.
- [21] Noakes P.G., Miner J.H., Gautam M., Cunningham J.M., Sanes J.R., Merlie J.P., The renal glomerulus of mice lacking s-laminin/laminin β 2: nephrosis despite molecular compensation by laminin β 1. *Nat. Genet.* 10 (1995) 400–406.
- [22] Patton B.L., Chiu A.Y., Sanes J.R., Synaptic laminin prevents glial entry into the synaptic cleft. *Nature* 393 (1998) 698–701.
- [23] Patton B.L., Miner J.H., Chiu A.Y., Sanes J.R., Localization, regulation and function of laminins in the neuromuscular system of developing, adult and mutant mice. *J. Cell Biol.* 139 (1997) 1507–1521.
- [24] Sanes J.R., Apel E.D., Gautam M., Glass D., Grady R.M., Martin P.T., Yancopoulos G.D., Agrin receptors at the skeletal neuromuscular junction. *Ann. N.Y. Acad. Sci.* (1998), in press.
- [25] Sanes J.R., Lichtman J.W., Development of the vertebrate neuromuscular junction. *Annu. Rev. Neurosci.* (1999), in press.
- [26] Zhou H., Grady R.M., Sanes J.R., Muscular dystrophy and impaired aggregation of acetylcholine receptors in α -dystrobrevin-deficient mutant mice. *Neurosci. Abstr.* (1998), in press.

Transcriptional pathways for synapse-specific, neuregulin-induced and electrical activity-dependent transcription

Larry Fromm and Steven J. Burden

Molecular Neurobiology Program, Skirball Institute, NYU Medical Center, 540 First Avenue, New York, NY 10016, USA

Abstract — Innervation-dependent expression of acetylcholine receptor (AChR) genes in skeletal muscle is mediated by multiple transcriptional pathways. One pathway leads to activation of AChR genes selectively in synaptic nuclei and requires an Ets binding site that binds GABP. A second pathway leads to repression of AChR transcription in nuclei throughout the myofiber and requires inactivation of E-box-binding proteins, including myogenic bHLH proteins. Taken together, these studies indicate that separate pathways regulate innervation-dependent transcription. (©Elsevier, Paris)

Résumé — Les voies de la transcription synapse-spécifique, induit par la neuréguline et dépendant de l'activité électrique. L'expression des gènes codant les récepteurs à l'acétylcholine (RACH) dépendante de l'innervation dans le muscle squelettique est engendrée par de multiples voies transcriptionnelles. Une de ces voies aboutit à l'activation des gènes RACH sélectivement dans les noyaux synaptiques et nécessite la présence de protéines liant les sites Ets, y compris GABP. Une deuxième voie aboutit à la répression de la transcription des RACH dans les noyaux dans toute la myofibre et nécessite l'inactivation des protéines liant la boîte E. (©Elsevier, Paris)

acetylcholine receptors / neuromuscular synapses / Ets proteins / GABP

1. Introduction

Localization of acetylcholine receptors (AChRs) to neuromuscular synapses is mediated by multiple transcriptional pathways. One pathway, which is stimulated by a signal that is concentrated at synaptic sites, induces expression of AChR genes in myofiber nuclei that are positioned near the synaptic site (for reviews see [4, 5, 8, 10]). Neuregulin (NRG) is the best candidate for the extracellular signal that induces synapse-specific gene expression, since NRG activates AChR gene expression in cultured muscle cells and NRG and its receptors are concentrated at synaptic sites [2, 7, 9, 11, 14, 17, 25]. A second pathway, which depends upon propagated electrical activity in the myofiber, represses AChR gene expression in nuclei throughout the myofiber (for review see [10]). These two pathways act together to restrict AChR expression to synaptic sites.

181 bp of 5' flanking DNA from the AChR δ -subunit gene are sufficient to confer both synapse-specific and electrical activity-dependent transcription in transgenic mice [19, 21]. These two aspects of innervation-dependent expression, however, are controlled by distinct regulatory elements since an E-box is required for electrical activity-dependent but not synapse-specific transcription and an Ets-binding site is required for synapse-specific but not electrical activity-dependent transcription [21] (Fromm and Burden, submitted). Taken together, these results indicate that separate transcriptional pa-

thways mediate synapse-specific and electrical activity-dependent gene expression.

2. Materials and methods

Myoblasts were transfected, induced to differentiate into myotubes, and the stably transfected cells were treated with NRG for 2 days [11, 18] (Fromm and Burden, submitted). The amount of hGH secreted from treated and untreated cells was measured by a radioimmunoassay [11].

Nuclear extracts from Sol8 myotubes were incubated with a radiolabeled oligonucleotide probe (nucleotides –62 to –47 in the AChR δ subunit gene) [11, 18] (Fromm and Burden, submitted), and complexes were resolved by electrophoresis. The specificity of the binding reaction was determined by the addition of unlabelled competitor DNA to the reaction mixture.

Antibodies to Ets proteins, which were either kindly supplied by colleagues or purchased from Santa Cruz Biotechnology, were incubated with nuclear extracts prior to the addition of the labeled probe [11, 18] (Fromm and Burden, submitted).

We stained muscles from transgenic mice carrying AChR δ subunit-hGH transgenes with antibodies to hGH and Texas Red α -bungarotoxin [19, 21] (Fromm and Burden, submitted), and we measured the level of hGH, AChR δ subunit and actin mRNA expression by RNase protection [19, 21] (Fromm and Burden, submitted).

3. Results

181 bp of 5' flanking DNA from the AChR δ subunit gene are sufficient to confer synapse-specific transcription in transgenic mice and NRG-induced

transcription in cultured muscle cells [11, 19, 21]. Changeux and colleagues showed that a sequence (CGGAA; nucleotides -54 to -58) that conforms to a consensus binding site (C/AGGAA/T) for Ets proteins is important for synapse-specific expression [12]. We showed that this potential binding site for Ets proteins is required for NRG-induced transcription, since NRG induces a ~10-fold increase in gene expression from myotubes transfected with a wild-type AChR δ regulatory region (-1823 ± 25) but only a modest (1.8-fold) increase in gene expression from myotubes transfected with an AChR δ regulatory region containing mutations (CAAAA) in nucleotides that are critical for binding Ets proteins (Fromm and Burden, submitted).

To determine whether the sequence requirements for NRG-induced transcription conform to the sequence specificity for binding Ets proteins, we transfected myotubes with gene fusions containing a C, A or T at the 5' end of the core Ets-binding site. We found that mutation of the C to an A results in a reduced but substantial (5-fold) response to NRG, whereas mutation of the C to a T results in a weak NRG response (1.7-fold) (Fromm and Burden, submitted). Since Ets proteins bind sequences containing a C or an A, but not a T (C/AGGAA/T) at the 5' end of a core Ets-binding site [25], these results are consistent with the idea that the NRG-response element (NRE) binds Ets proteins and that this binding is important for NRG-induced expression of the AChR δ subunit gene.

We found that mutation of the NRE reduces expression from untreated cells by ~7-fold, and these results suggest that this NRE is also required for maximal expression from myotubes not treated with NRG (Fromm and Burden, submitted). Taken together with studies showing that muscle cells synthesize NRG [14, 16], these results raise the possibility that AChR gene expression from untreated cells is nevertheless NRG-dependent and regulated by autocrine signaling.

We analyzed synapse-specific transcription by producing transgenic mice carrying gene fusions between the mouse AChR δ subunit gene and the hGH gene [19, 21]. Because hGH is processed in the endoplasmic reticulum and Golgi apparatus and because these organelles are closely associated with nuclei, it is possible to infer the nuclear source of hGH transcription by studying the spatial pattern of intracellular hGH using immunohistochemistry. We produced transgenic mice carrying wild-type or mutated AChR δ subunit-hGH gene fusions and showed that transgenes containing a mutation in the NRE, unlike wild-type transgenes, are not expressed selectively from synaptic nuclei, demonstrating that the

NRE is required for synaptic expression (Fromm and Burden, submitted).

An absence of synaptic expression could be owing to a specific role for this NRE in synapse-specific transcription or to a requirement for this NRE in transcription per se. We denervated muscle from mice carrying transgenes with a mutated NRE and showed that the mutant transgenes, like wild-type transgenes are induced following denervation (Fromm and Burden, submitted). These results demonstrate that the transgenes containing a mutation in this NRE are transcriptionally competent and not simply inactive, indicating that the NRE is required specifically for synapse-specific transcription. Further, these results show that the NRE is not required for electrical activity-dependent gene expression.

E-boxes are important regulatory elements for activating muscle genes, including AChR subunit genes [15], during myogenesis. We asked whether E-boxes in the AChR δ subunit gene may therefore be required for synapse-specific expression. We produced transgenic mice carrying AChR δ subunit-hGH gene fusions with a mutation in the E-box near the transcription start site and showed that these transgenes, like wild-type transgenes are expressed selectively from synaptic nuclei, demonstrating that this E-box is not required for synaptic expression [21]. This E-box, unlike the NRE, however, is required for electrical activity-dependent transcription, since transgenes with a mutated E-box, unlike wild-type transgenes or transgenes with a mutated NRE, are not induced following denervation [3, 21]. These results demonstrate that this E-box is required specifically for electrical activity-dependent transcription and are consistent with the idea that electrical activity represses myogenic bHLH proteins either by decreasing their expression and/or activity.

We found that protein(s) in myotube nuclear extracts bind an oligonucleotide probe containing the NRE. Binding is competed by an excess of unlabelled, competitor DNA containing a wild-type NRE but not by competitor DNA containing mutations in nucleotides that are important for NRG responsiveness (Fromm and Burden, submitted). Thus, there is a good correlation between nucleotides required for NRG-responsiveness and protein-binding.

NRG stimulation does not appear to recruit the protein(s) to the NRE, since a similar protein/DNA complex is detected with nuclear extracts from untreated myotubes (Fromm and Burden, submitted). These data favor the idea that NRG increases the transcriptional activity rather than the DNA-binding activity of the protein(s) that bind to this NRE.

We incubated nuclear extracts from NRG-stimulated myotubes with antibodies to Ets proteins to determine whether known Ets proteins are present in

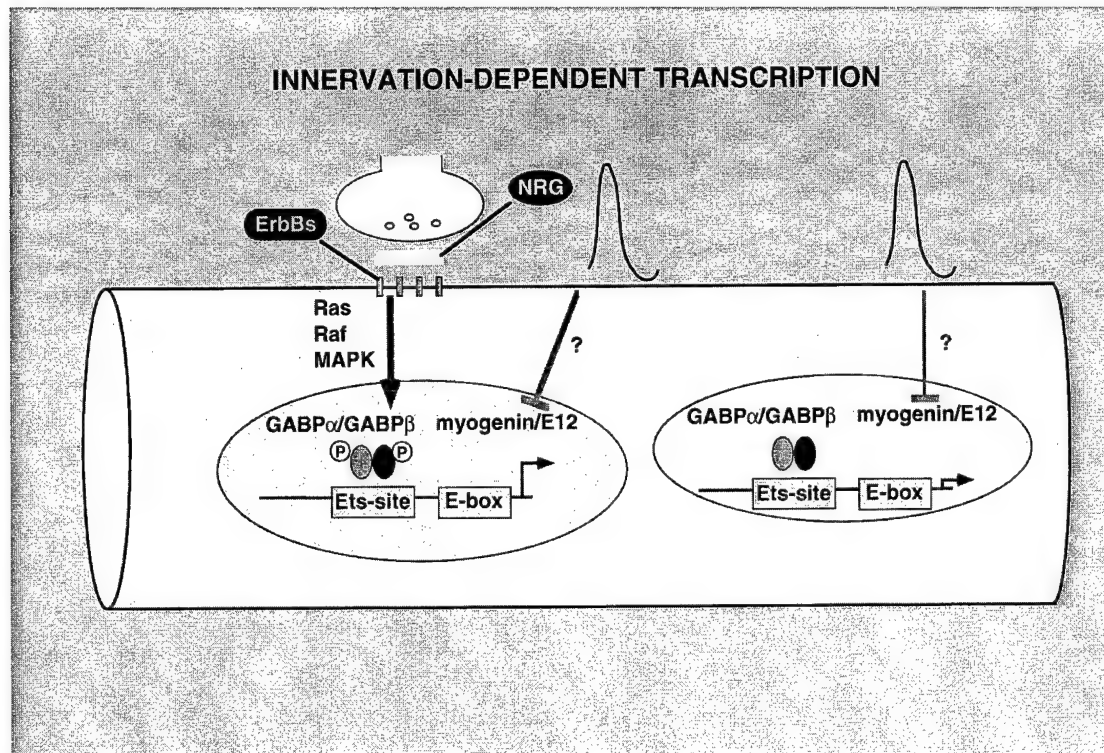


Figure 1. Separate pathways regulate synapse-specific and electrical activity-dependent transcription. NRG, which is present in the synaptic basal lamina, activates ErbB receptors in the postsynaptic membrane and leads to activation of a Ras signaling pathway. Our experiments suggest that this pathway leads to activation of GABPα/GABPβ and an increase in AChR gene expression selectively in synaptic nuclei. The pathway activated by electrical activity is thought to result in a decrease in the activity and/or abundance of transcriptional activators that bind E-boxes, such as myogenin and E12, leading to a decrease in AChR gene expression in nuclei throughout the muscle fiber.

the protein/DNA complex detected in the EMSA. We found that antibodies against either GABPα, an Ets protein, or GABPβ, a protein which lacks an Ets domain but dimerizes with GABPα [23], both inhibit the formation of the protein/DNA complex and super-shift the complex (Fromm and Burden, submitted). In contrast, antibodies against eight other Ets proteins, four of which are known to be expressed in skeletal muscle cells, fail to inhibit formation of the protein/DNA complex (Fromm and Burden, submitted). These results indicate that GABPα is the major, if not the only Ets protein in this complex and that a complex of GABPα/GABPβ binds to the NRE.

4. Discussion

An Ets site in the AChR δ subunit gene binds GABP and is required for transcriptional induction of the AChR gene by NRG and for synapse-specific gene expression in transgenic mice. These results

provide further evidence for the idea that NRG is a signal for synapse-specific transcription and suggest that GABPα/GABPβ respond to NRG signaling by stimulating transcription of the AChR δ subunit gene.

NRG stimulation results in activation of a Ras/Raf/MAP kinase signaling cascade, and both Ras and MAP kinase are required for induction of AChR genes by NRG [1, 20, 22]. Ets proteins, including GABPα, are known targets of Ras/MAP kinase signaling, and in vitro studies have shown that Ets proteins, including GABPα, can be phosphorylated directly by MAP kinase. Taken together, these studies raise the possibility that GABPα/GABPβ binds the NRE in the AChR δ subunit gene and that Ras signaling leads to transcriptional activation of GABP and induction of AChR genes.

GABP is present in myotube nuclear extracts and binds to the NRE in the AChR δ subunit promoter. Although Elf-1, Elf-2, ERP, NERF, ER81, Ets-2 and GABP are known to be expressed in skeletal muscle, GABPα/GABPβ appears to be the major, if not only

Ets protein that binds the NRE. The apparent preference for GABP could be owing to a greater abundance of GABP than other Ets proteins in skeletal muscle or to a higher affinity of GABP for the NRE.

An E-box is important for electrical activity-dependent but not synapse-specific transcription of AChR genes [3, 21]. In contrast, the NRE is required for synapse-specific but not electrical activity-dependent transcription (Fromm and Burden, submitted). Taken together, these studies indicate that separate pathways regulate innervation-dependent transcription: an electrical activity-dependent pathway that leads to a decrease in the activity and/or expression of myogenic bHLH proteins and a synapse-specific pathway that leads to the activation of GABP α /GABP β .

Acknowledgments

This work was supported by research grants from the National Institutes of Health (NS27963) and the Muscular Dystrophy Association and by a postdoctoral fellowship from the NIH to L.F.

References

- [1] Altiok N., Altiok X., Changeux J.P., Heregulin-stimulated acetylcholine receptor gene expression in muscle: requirement for MAP kinase and evidence for a parallel inhibitory pathway independent of electrical activity, *EMBO J.* 16 (1997) 717–725.
- [2] Altiok N., Bessereau J.L., Changeux J.P., ErbB3 and ErbB2/neu mediate the effect of heregulin on acetylcholine receptor gene expression in muscle: differential expression at the endplate, *EMBO J.* 14 (1995) 4258–4566.
- [3] Bessereau J.L., Stratford-Perricaudet L.D., Piette J., LePoupon C., Changeux J.P., In vivo and in vitro analysis of electrical activity-dependent expression of muscle acetylcholine receptor genes using adenovirus, *Proc. Natl. Acad. Sci. USA* 91 (1994) 1304–1308.
- [4] Burden S.J., The formation of neuromuscular synapses, *Genes Dev.* 12 (1998) 133–148.
- [5] Duclert A., Changeux J.P., Acetylcholine receptor gene expression at the developing neuromuscular junction, *Physiol. Rev.* 75 (1995) 339–368.
- [6] Duclert A., Savatier N., Schaeffer L., Changeux J.P., Identification of an element crucial for the sub-synaptic expression of the acetylcholine receptor epsilon-subunit gene, *J. Biol. Chem.* 271 (1996) 17433–17438.
- [7] Falls D.L., Rosen K.M., Corfas G., Lane W.S., Fischbach G.D., ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family, *Cell* 72 (1993) 801–815.
- [8] Fischbach G.D., Rosen K.M., A neuromuscular junction neu-regulin, *Annu. Rev. Neurosci.* 20 (1997) 429–458.
- [9] Goodearl A.D., Yee A.G., Sandrock A.W., Corfas G., Fischbach G.D., ARIA is concentrated in the synaptic basal lamina of the developing chick neuromuscular junction, *J. Cell Biol.* 130 (1995) 1423–1434.
- [10] Hall Z.W., Sanes J.R., Synaptic structure and development: the neuromuscular junction, *Cell/Neuron* 72/10 (Suppl.) (1993) 99–121.
- [11] Jo S.A., Zhu X., Marchionni M.A., Burden S.J., NRGs are concentrated at nerve-muscle synapses and activate Ach-receptor gene expression, *Nature* 373 (1995) 158–161.
- [12] Koike S., Schaeffer L., Changeux J.P., Identification of a DNA element determining synaptic expression of the mouse acetylcholine receptor delta-subunit gene, *Proc. Natl. Acad. Sci. USA* 92 (1995) 10624–10628.
- [13] LaMarco K., Thompson C.C., Byers B.P., Walton E.M., McKnight S.L., Identification of Ets- and Notch-related subunits in GA binding protein, *Science* 253 (1991) 789–792.
- [14] Moscoso L.M., Chu G.C., Gautam M., Noakes P.G., Merlie J.P., Sanes J.R., Synapse-associated expression of an acetylcholine receptor-inducing protein, ARIA/hergulin, and its putative receptors, ErbB2 and ErbB3, in developing mammalian muscle, *Dev. Biol.* 172 (1995) 158–169.
- [15] Piette J., Bessereau J.L., Huchet M., Changeux J.P., Two adjacent MyoD1-binding sites regulate expression of the acetylcholine receptor α -subunit gene, *Nature* 345 (1990) 353–355.
- [16] Rimer M., Cohen I., Lømo T., Burden S.J., McMahan U.J., Neural agrin induces aggregation of muscle-derived ARIA and its receptors erbB2 and erbB3, in adult rat soleus muscle, *Mol. Cell. Neurosci.*, in press.
- [17] Sandrock A.W., Dryer S.E., Rosen K.M., Gozani S.N., Kramer R., Theil L.E., Fischbach G.D., Maintenance of acetylcholine receptor number by neuregulins at the neuromuscular junction in vivo, *Science* 276 (1997) 599–603.
- [18] Simon A.M., Burden S.J., An E box mediates activation and repression of the acetylcholine receptor δ -subunit gene during myogenesis, *Mol. Cell. Biol.* 13 (1993) 5133–5150.
- [19] Simon A.M., Hoppe P., Burden S.J., Spatial restriction of AChR gene expression to subsynaptic nuclei, *Development* 114 (1992) 545–553.
- [20] Si J., Luo Z., Mei L., Induction of acetylcholine receptor gene expression by ARIA requires activation of mitogen-activated protein kinase, *J. Biol. Chem.* 271 (1996) 19752–19759.
- [21] Tang J., Jo S.A., Burden S.J., Separate pathways for synapse-specific and electrical activity-dependent gene expression in skeletal muscle, *Development* 120 (1994) 1799–1804.
- [22] Tansey M.G., Chu G.C., Merlie J.P., ARIA/HRG regulates AChR ϵ subunit gene expression at the neuromuscular synapse via activation of phosphatidylinositol 3-kinase and Ras/MAPK pathway, *J. Cell Biol.* 134 (1996) 465–476.
- [23] Thompson C.C., Brown T.A., McKnight S.L., Convergence of Ets- and Notch-related structural motifs in a heteromeric DNA binding complex, *Science* 253 (1991) 762–768.
- [24] Wasyluk B., Hahn S.L., Giovane A., The Ets family of transcription factors, *Eur. J. Biochem.* 211 (1993) 7–18.
- [25] Zhu X., Lai C., Thomas S., Burden S.J., Neuregulin receptors, erbB3 and erbB4, are localized at neuromuscular synapses, *EMBO J.* 14 (1995) 5842–5848.

Targeting of acetylcholine receptor and 43 kDa rapsyn to the postsynaptic membrane in *Torpedo marmorata* electrocyte

Fabrizia Bignami, Gilles Camus, Sophie Marchand, Lise Bailly,
Françoise Stetzkowski-Marden, Jean Cartaud

*Biologie Cellulaire des Membranes, Institut Jacques-Monod, CNRS, Universités Paris 6 et Paris 7,
2, place Jussieu, 75251 Paris cedex 05, France*

Abstract — In this study we have investigated the intracellular routing of two major components of the postsynaptic membrane in *Torpedo* electrocytes, the nicotinic acetylcholine receptor and the extrinsic 43 kDa protein rapsyn, and of a protein from the non-innervated membrane, the Na^+, K^+ ATPase. We isolated subpopulations of post-Golgi vesicles (PGVs) enriched either in AChR or in Na^+, K^+ ATPase. Rapsyn was associated to AChR-containing PGVs suggesting that both AChR and rapsyn are targeted to intracellular organelles in the secretory pathway before delivery to the postsynaptic membrane. In vitro assays further show that rapsyn-containing PVGs do bind more efficiently to microtubules compared to Na^+, K^+ ATPase-enriched PVGs. These data provide evidence in favor of the contribution of the secretory pathway to the delivery of synaptic components. (©Elsevier, Paris)

Résumé — Adressage du récepteur de l'acétylcholine et de la rapsyne à la membrane postsynaptique de l'électrocyte de torpille. Nous avons étudié le trafic intracellulaire de deux protéines synaptiques : le récepteur de l'acétylcholine et la rapsyne-43kDa dans l'électrocyte de torpille ainsi que l'ATPase Na^+, K^+ , la protéine majeure de la membrane non-innervée. Nous avons isolé différentes sous-populations de vésicules post-golgiennes, enrichies respectivement en AChR et en ATPase Na^+, K^+ . La rapsyne est associée spécifiquement à des vésicules riches en AChR, ce qui suggère son adressage à la voie d'exocytose avant son adressage à la membrane plasmique. La liaison *in vitro* des vésicules contenant la rapsyne à des microtubules suggère la contribution des voies d'exocytose dans l'adressage direct des protéines synaptiques. (©Elsevier, Paris)

acetylcholine receptor / rapsyn / intracellular trafficking / microtubules / synaptogenesis

1. Introduction

Accumulation of acetylcholine receptors (AChRs) and other synaptic proteins at the neuromuscular junction (NMJ) results from several regulatory mechanisms. In situ hybridization and promoter studies in transgenic mice have demonstrated that the restricted distribution of several synaptic proteins at the NMJ results in part from differential transcription in synaptic vs. extrasynaptic regions of the muscle fiber (reviewed in [4]). This mechanism suggests a local biosynthesis of synaptic components, as well as a polarized sorting in the exocytic pathway, the latter event being a key feature of polarized cells which achieve the differentiation of plasma membrane domains by way of protein sorting in the Golgi apparatus or in the endosomes (reviewed in [9]).

In addition to the AChR, the mature post-synaptic membrane accommodates a host of extrinsic proteins. Among them, the 43 kDa rapsyn is particularly important since it plays a major role in the regulation of the clustering and in the maintenance of high densities of AChRs at the synapse. Rapsyn lacks a signal sequence for membrane insertion but possesses an amino-terminal myristoylation site [2] responsible in part for its targeting to the plasma membrane [13].

As such, rapsyn could possibly be translated on free polysomes, acylated and inserted into the plasma membrane. Recent evidence shows, however, that processing and targeting of several acylated proteins depends on a functional secretory pathway [5, 11]. In this line, rapsyn may associate with intracellular organelles followed by vesicular transport to the plasma membrane.

The electrocyte of the electric ray *Torpedo marmorata* has long been used as a model system for the study of cholinergic synapses. Here, we have taken advantage of its intrinsic polarity to study the intracellular trafficking and the delivery of AChR and rapsyn to the innervated membrane, compared to that of the Na^+, K^+ ATPase, a marker of the non-innervated membrane.

2. Materials and methods

2.1. Animals

Torpedoes (*Torpedo marmorata*) were obtained from the Stations de Biologie Marine, Roscoff (Université Paris 6) and Arcachon (Université Bordeaux I), France.

2.2. Antibodies

Polyclonal antibodies directed against *Torpedo* Na⁺,K⁺ ATPase and AChR were obtained in our laboratory [1]. A monoclonal antibody directed against rapsyn (mAb 1234) was kindly provided by Dr. S. Froehner [12]. A monoclonal antibody directed against glutaminated α -tubulin (mAb GT335) was a generous gift of Dr. P. Denoulet [18]. Monoclonal antibodies directed against cytoplasmic epitopes of AChR α -subunit (mAbs 111 and 155) were kindly provided by Dr. S. Tzartos [17].

2.3. Immunoisolation of post-Golgi vesicles enriched in AChR, rapsyn or Na⁺,K⁺ ATPase

Crude post-Golgi vesicles were isolated following the protocol described in Camus et al. [1]. The AChR, rapsyn and Na⁺,K⁺ ATPase-enriched post-Golgi vesicles (V_R, V₄₃ and V_A) were immunopurified on magnetic beads (Dynabeads M-280, Dynal AS Oslo, Norway) coated by mAbs 111/155, mAb 1234 and polyclonal anti- Na⁺,K⁺ ATPase, respectively (see Camus et al. [1] for details). For biochemical analysis by SDS-PAGE and Western blotting, beads were directly resuspended in Laemmli buffer [10].

2.4. Mono-dimensional SDS-PAGE and Western blotting

Proteins from various membranes preparations were separated on 10% monodimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a slab cell (Mini Protean II, BioRad Richmond CA, USA). After separation, proteins were electrotransferred onto nitrocellulose paper (Schleicher and Schuell, Dassel, Germany) according to Towbin et al. [16]. Immunoblot experiments were performed as described elsewhere [3] and the immunodetections were achieved using the chemiluminescent reaction (ECL, Amersham, UK) using X-ray films.

2.5. Microtubule binding assays

Phosphocellulose (PC)-purified pig brain tubulin was polymerized with 20 μ M taxol for 30 min at 37°C in PEMT (80 mM K-Pipes, 1 mM EGTA, 1 mM MgCl₂, 20 μ M taxol, pH 6.8). Fragments of microtubules obtained by sonication were incubated at room temperature with AChR-enriched post-Golgi vesicles immuno-isolated on magnetic beads (see above), in PEMT in a final volume of 0.5 mL. Cytosol from *Torpedo* electric organ was added at a final concentration of 3 mg/mL and parallel experiments without cytosol were performed as control. Magnetic beads were collected, washed once in PEMT, and treated for electron microscopy analysis as described below. Quantitative microtubule-binding assay was performed as follows: microtubules (see above) were immuno-adsorbed on magnetic beads using mAb GT335. Incubations with crude PGVs in the presence of cytosol (3 mg/mL in PEMT) or with 0.3% BSA in the control were performed at room temperature. After two gentle washes, immunoblot analysis of the bound vesicles was carried out as described.

2.6. Electron microscopy and immunogold labeling

Vesicles immuno-isolated on magnetic beads were labeled in batch using secondary antibodies conjugated to 10 nm gold particles [15] (Slot and Geuze, 1985) and then fixed in 2.5% glutaraldehyde, 0.1% tannic acid in 0.1 M cacodylate buffer, pH 7.4, and post-fixed with 1% osmium tetroxide. Embedding in epoxy resin was performed after dehydration in a series of ethanol solutions. Thin sections were stained with 5% uranyl acetate and observed with a Philips CM12 electron microscope operating at 80 kV.

3. Results and discussion

3.1. Biochemical and immunocytochemical characterization of post-Golgi vesicles (PGVs) immunopurified with anti-AChR, anti-rapsyn and anti-ATPase antibodies

To unravel the exocytic pathway of synaptic proteins, we first isolated crude post-Golgi vesicles (PGVs) using sucrose equilibrium gradient centrifugation. As in other tissues, these vesicles have a buoyant density of 1.09–1.10 g/mL and a mean diameter of 80–100 nm [1]. Distinct populations of PGVs were purified, by using immunomagnetic beads, on the basis of their content in AChR, rapsyn and Na⁺,K⁺ ATPase, the major components of the innervated and non-innervated membrane domains of the electrocyte, respectively. The protein content of each population was determined using Western blotting experiments. *Figure 1* shows that PGVs isolated either with anti-AChR or anti-rapsyn antibodies were almost devoid of Na⁺,K⁺ ATPase. Conversely, PGVs isolated with anti-Na⁺,K⁺ ATPase were devoid of AChR or rapsyn. These data suggest that these classes of PGVs result from a sorting in the Golgi apparatus. Surprisingly, AChR-purified PGVs contained low and variable amounts of rapsyn, while rapsyn-purified PGVs always contained equal amounts of AChR and rapsyn (*figure 1*). This indicates that several subpopulations of AChR-containing PGVs contain variable amounts of rapsyn. These populations of PGVs most likely result from a gradient of maturation of AChR-containing PGVs to which rapsyn molecules bind, after budding from the *trans* Golgi network. This could be accounted for by the presence of coat proteins necessary for the budding of PGVs from the *trans* Golgi compartment, assuming that the association of rapsyn occurs only after uncoating. In agreement with this hypothesis, Golgi fractions purified from *Torpedo* electrocytes usually have a low rapsyn content (not shown).

Electron microscope analysis was used to show that the immunopurified membranes corresponded to uncoated vesicles and not to Golgi stacks (*figure*

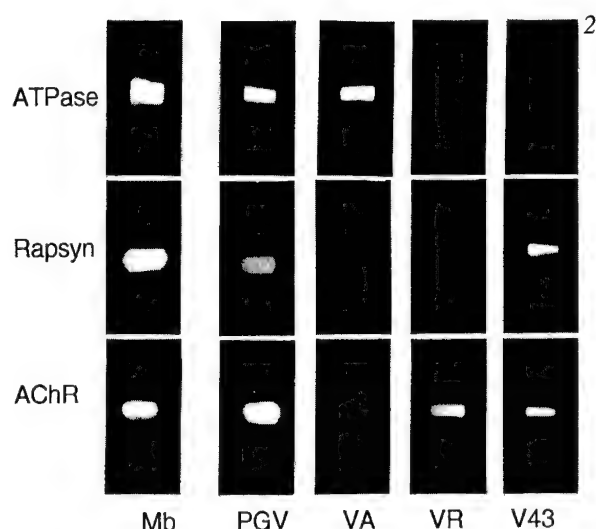


Figure 1. Biochemical characterization of PGV fractions. The content of immunopurified PGV fractions (V_A , V_R and V_{43}) was analyzed by immunoblotting after SDS-PAGE using polyclonal anti- Na^+ , K^+ ATPase and anti-AChR antibodies and mAb 1234 anti-rapsyn, and compared to crude *Torpedo* plasma membranes (Mb) and crude PGVs. To allow quantitative comparison between different fractions, the immunodetection of the proteins in a given fraction was carried out on the same nitrocellulose strip. All blots were exposed for ECL detection during the same period of time. V_A contained very low amounts of AChR and rapsyn; V_R contained traces of Na^+ , K^+ ATPase and variable but always low amounts of rapsyn, whereas V_{43} contained low amounts of Na^+ , K^+ ATPase but equal amounts of AChR and rapsyn.

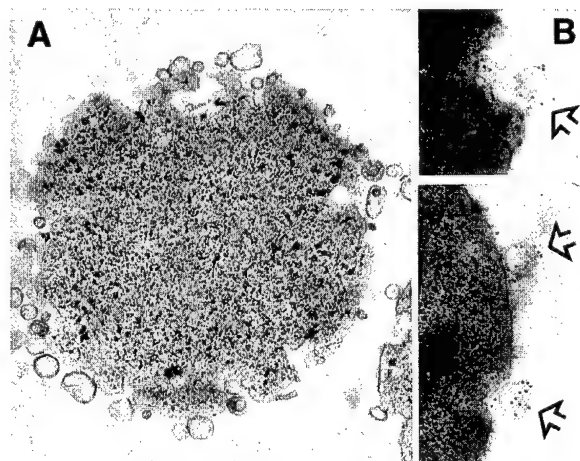


Figure 2. EM analysis of AChR and rapsyn-enriched PGV fractions. Uncoated vesicles were visualized around magnetic beads coated with anti-AChR mAbs (A, magnification = 34 000). Vesicles immunopurified with anti-rapsyn mAb displayed a similar aspect (not shown). Immunogold labeling with anti AChR antibodies, revealed that most of these vesicles contained AChR protein (arrows in B; magnification = 50 000).

A). Immunogold labeling confirmed that most of the rapsyn-purified vesicles contained AChR (figure 2B).

3.2. Role of microtubules in the intracellular routing of postsynaptic proteins

In polarized cells, microtubules are involved in the direct delivery of newly synthesized surface proteins, by facilitating their intracellular routing to the apical (and also basolateral) domains and by limiting missorting (see [6] and references therein). Previous data from our laboratory and others have reported the presence of a specialized meshwork of stable microtubules in the subneural sarcoplasm in skeletal muscle fibers [7, 14]. Similarly, a polarized microtubule network is localized in the subneural region of the electrocyte [8]. To address the question of the role of microtubules in the intracellular trafficking of synaptic proteins, we have carried out in vitro binding experiments with microtubules polymerized from pig brain tubulin and PGVs. In a first approach, AChR-containing PGVs immunopurified on magnetic beads were tested for their competence to bind microtubules. As monitored by thin-section electron microscopy, this assay reveals the presence of microtubules at the surface of AChR-containing PGVs (figure 3A). In a second series of experiments, microtubules attached to magnetic beads were used in an in vitro binding assay including crude PGVs and electrocyte cytosol. The protein content of the bound vesicles in this assay was analyzed using Western blotting (figure 3B). By comparison with the crude PGV fraction (or the binding in the absence of cytosol), the rapsyn/ Na^+ , K^+ ATPase ratio in the fraction bound to microtubules was increased in the presence of cytosol. These data show that the subpopulation of PGVs enriched in rapsyn binds to microtubules in a cytosol-dependent manner. Given the vectorial organization of the microtubular network in electrocytes, this selective binding is likely to favor the proper delivery of synaptic proteins.

4. Concluding remarks

In this study, we have investigated the intracellular routing of two significant proteins of the postsynaptic membrane, the nicotinic acetylcholine receptor and rapsyn. The AChR being an intrinsic membrane component, it was reasonable to assume that it follows the secretory pathway. In this line, we isolated subpopulations of post-Golgi vesicles that were enriched either in AChR or in Na^+ , K^+ ATPase, an intrinsic protein from the non-innervated membrane. These vesicles are likely to result from a sorting in the Golgi apparatus. The targeting of

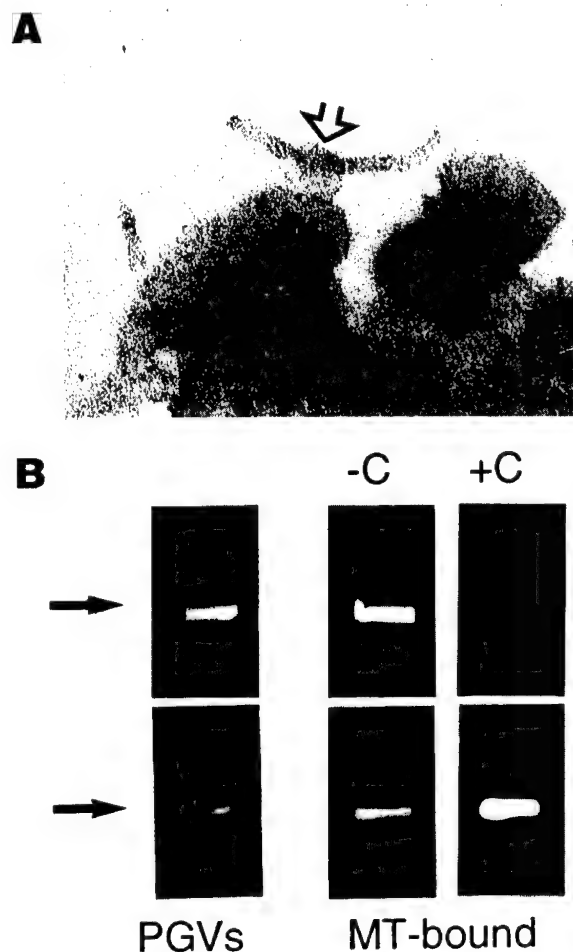


Figure 3. AChR and rapsyn-containing PGVs bind to microtubules. **A.** Vesicles immunopurified with anti-AChR coated beads are able to bind microtubules (arrow, magnification = 65 000). **B.** In vitro binding assay was carried out to identify PGV subpopulations able to bind to microtubules in the presence of cytosol. Taxol-polymerized microtubules were attached to magnetic beads. The protein content of the bound vesicles was analyzed using Western blotting. Immunoblotting analysis of vesicles recovered with microtubules revealed that, in the presence of cytosol (+C), rapsyn-containing vesicles are predominant. In the absence of cytosol (-C), non-specific binding of all classes of PGVs probably accounted for the high Na^+ , K^+ ATPase/rapsyn ratio, a pattern similar to that observed for crude PGVs.

rapsyn, an extrinsic component of the postsynaptic membrane was also studied. As a myristoylated protein, it is synthesized on free polysomes and could either be targeted to the postsynaptic membrane, independently of the AChR, or to intracellular compartments en route to the plasma membrane. We

showed that rapsyn associated to AChR-containing PGVs, suggesting that AChR and rapsyn are targeted to intracellular organelles in the secretory pathway before delivery to the postsynaptic membrane. Since we isolated PGVs containing AChR and a low amount of rapsyn as well as PGVs containing both proteins, it is possible that rapsyn associates with AChR-containing vesicles only after their budding from the TGN and uncoating. As in other polarized cells, a vectorial organization of the microtubular network, which is thought to facilitate vesicle trafficking between the Golgi apparatus and the cell surface, was observed in *Torpedo* electrocytes [1, 8]. The present in vitro assays further show that rapsyn-containing PGVs do bind more efficiently to microtubules, compared to Na^+ , K^+ ATPase-enriched PGVs. Considered together, these data bring strong evidence that the secretory pathway contributes to the localization of synaptic proteins.

Acknowledgments

We thank M.A. Ludosky for advice in electron microscopy and Dr J. Massoulié for the critical reading of the manuscript. This work was supported by CNRS, Universités Paris 6 and Paris 7 and the Association Française contre les Myopathies. F. Bignami is recipient of a Post Doctoral grant from the Fondazione 'Inst. Pasteur-Fondazione Cenci Bolognetti'.

References

- [1] Camus G., Jasmin B.J., Cartaud J., Polarized sorting of nicotinic acetylcholine receptor to the postsynaptic membrane in *Torpedo* electrocyte, *Eur. J. Neurosci.* 10 (1998) 839–852.
- [2] Carr C., Tyler A.N., Cohen J.B., Myristic acid is the NH_2 -terminal blocking group of the 43 kDa protein of *Torpedo* nicotinic postsynaptic membranes, *FEBS Lett.* 243 (1989) 65–69.
- [3] Cartaud A., Stetzkowski-Marden F., Cartaud J., Identification of dystrophin-binding protein(s) in membranes from *Torpedo* electrocyte and rat muscle, *J. Biol. Chem.* 268 (1993) 13019–13022.
- [4] Duclert A., Changeux J.P., Acetylcholine receptor gene expression at the developing neuromuscular junction, *Physiol. Rev.* 75 (1995) 339–368.
- [5] Gonzalo S., Linder M.E., SNAP-25 palmitoylation and plasma membrane targeting require a functional secretory pathway, *Mol. Biol. Cell.* 9 (1998) 589–597.
- [6] Grindstaff K.K., Bacallao R.L., Nelson W.J., Apiconuclear organization of microtubules does not specify protein delivery from the trans-Golgi network to different membrane domains in polarized epithelial cells, *Mol. Biol. Cell.* 9 (1998) 685–699.
- [7] Jasmin B.J., Changeux J.P., Cartaud J., Compartmentalization of cold-stable and acetylated microtubules in the subsynaptic domain of chick skeletal muscle fiber, *Nature* 344 (1990) 673–675.

- [8] Jasmin B.J., Changeux J.P., Cartaud J., Organization and dynamics of microtubules in *Torpedo marmorata* electrocyte: Selective association with specialized domains of the post-synaptic membrane, *Neuroscience* 43 (1991) 151–162.
- [9] Keller P., Simons K., Post-Golgi biosynthetic trafficking, *J. Cell. Sci.* 110 (1997) 3001–3009.
- [10] Laemmli U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T₄, *Nature* 227 (1970) 680–685.
- [11] Liu Y., Fischer D.A., Storm D.R., Intracellular sorting of neuromodulin (GAP-43) mutants modified in the membrane targeting domain, *J. Neurosci.* 14 (1994) 5807–5817.
- [12] Peng, H.B., Froehner S.C., Association of the postsynaptic 43-kD protein with newly formed acetylcholine receptor clusters in cultured muscle cells, *J. Cell Biol.* 100 (1985) 1698–1705.
- [13] Phillips W.D., Maimone M.M., Merlie J.P., Mutagenesis of the 43-kDa postsynaptic protein defines domains involved in plasma membrane targeting and AChR clustering, *J. Cell. Biol.* 115 (1991) 1713–1723.
- [14] Rahkila P., Väänänen K., Saraste J., Metsikkö K., Endoplasmic reticulum to Golgi trafficking in multinucleated skeletal muscle fibers, *Exp. Cell. Res.* 234 (1997) 452–464.
- [15] Slot, J.W., Geuse H.J., A new method of preparing gold probes for multiple labeling cytochemistry, *Eur. J. Cell Biol.* 38 (1985) 87–93.
- [16] Towbin H., Staehelin T., Gordon J., Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc. Natl. Acad. Sci. USA* 76 (1979) 4350–4354.
- [17] Tzartos S.J., Lindstrom J.M., Monoclonal antibodies used to probe acetylcholine receptor structure: localization of the main immunogenic region and detection of similarities between subunits, *Proc. Natl. Acad. Sci. USA* 77 (1980) 755–759.
- [18] Wolff A., de Néchaud B., Chillet D., Mazarguil H., Desbruyères E., Audebert S., Eddé B., Gros F., Denoulet P., Distribution of glutamylated α and β -tubulin in mouse tissues using a specific monoclonal antibody, GT335, *Eur. J. Cell Biol.* 59 (1992) 425–432.

Acetylcholinesterase: C-terminal domains, molecular forms and functional localization

Jean Massoulié, Alain Anselmet, Suzanne Bon, Eric Krejci,
Claire Legay, Nathalie Morel, Stéphanie Simon

*Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS-URA 1857, École Normale Supérieure,
46, rue d'Ulm, 75230 Paris cedex 05, France*

Abstract — Acetylcholinesterase (AChE) possesses short C-terminal peptides that are not necessary for catalytic activity. These peptides belong to different classes (R, H, T, S) and define the post-translational processing and targeting of the enzyme. In vertebrates, subunits of type H (AChE_H) and of type T (AChE_T) are the most important: AChE_H subunits produce glycolipid (GPI)-anchored dimers and AChE_T subunits produce hetero-oligomeric forms such as membrane-bound tetramers in the mammalian brain (containing a 20 kDa hydrophobic protein) and asymmetric collagen-tailed forms in neuromuscular junctions (containing a specific collagen, ColQ). The T peptide allows the formation of tetrameric assemblies with a proline-rich attachment domain (PRAD) of collagen ColQ. These complex molecular structures condition the functional localization of the enzyme in the supramolecular architecture of cholinergic synapses. (©Elsevier, Paris)

Résumé — Acétylcholinestérase : domaines C-terminaux, formes moléculaires et localisation fonctionnelle. L'acétylcholinestérase (AChE) possède de courts peptides C-terminaux qui ne sont pas indispensables pour l'activité enzymatique. Ces peptides, classés en différents types (R, H, T, S), déterminent la maturation et le devenir de l'enzyme. Chez les vertébrés, les sous-unités de type H (AChE_H) et de type T (AChE_T) sont les plus importantes, et produisent respectivement des dimères ancrés par un glycolipide (GPI) et des formes hétéro-oligomériques telles que les tétramères membranaires du cerveau des mammifères (comprenant une protéine hydrophobe de 20 kDa) et les formes asymétriques des jonctions neuromusculaires (comprenant un collagène spécifique, ColQ). Le peptide T permet la formation d'un assemblage tétramérique avec un domaine riche en prolines ('proline-rich attachment domain', PRAD) du collagène ColQ. Ces structures moléculaires complexes conditionnent la localisation fonctionnelle de l'enzyme dans l'architecture supramoléculaire des synapses cholinergiques. (©Elsevier, Paris)

acetylcholinesterase / anchoring / basal lamina / collagen

1. Introduction

An efficient hydrolysis of acetylcholine by cholinesterases depends on the precise localization of these enzymes, particularly in synaptic structures. The catalytic domain of cholinesterases is organized as an α/β hydrolase fold [1, 2], possessing the conformation of a globular protein without any trans-membrane or other anchoring feature. However, cholinesterase subunits present a variety of quaternary associations and can be attached to cell membranes or with extracellular structures in various ways, because of the presence of small C-terminal domains [3], as shown in *figure 1*.

2. Multiplicity of C-terminal domains: species and tissue distribution

The cholinesterase genes that have been analyzed so far contain three types of exons encoding distinct C-terminal domains, called H ('hydrophobic'), T ('tailed') and S ('soluble', or 'snake'). In addition, R ('readthrough') transcripts, which have been found in *Torpedo* and mammals, are not spliced after the last exon encoding the catalytic domain.

Invertebrates may possess one cholinesterase gene (*Drosophila*) [11], two genes like *Culex* [36] and amphioxus [37] or four cholinesterase genes like *Caenorhabditis* [38], but each gene seems to produce a single C-terminal domain. While insects and the pro-chordate amphioxus only possess cholinesterases of type H, the various genes of the nematode *Caenorhabditis* produce either subunits of type T, for the major *ace-1* gene [16], or of type H, for the less expressed *ace-2* gene, and probably also the minor *ace-3* and *ace-4* genes [38].

Vertebrates possess two cholinesterase genes, producing the enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) that differ in their substrate specificity: BChE hydrolyzes butyrylcholine, in addition to acetylcholine, while AChE is essentially inactive on the larger ester. This difference is now perfectly explained by the size of an acyl pocket in the active site of these enzymes, that accommodates a butyryl moiety in the case of BChE, but not in AChE because it is limited by two bulky phenylalanine side chains [39]. Both AChE and BChE genes produce subunits of type T [3]. In addition, the AChE genes of some species may

Type of subunit	Peptide sequence of C-terminal region	Molecular forms	Tissue distribution
R	<i>Torpedo</i> AChE: GNVFAPHMQRVTPAKTYHFGVIVAHLLLSLPTASDVRLA SSKWAHSDFL@S-RR@WESWGRIL [4] Rat AChE: GRRGVGQGMHKAARVGRTOGRKGKGRH [5] Mouse AChE: GRMWEQGMHKAARVGRTOGRKGKGRH [6] Human AChE: GMQGPAGSGWEEGSGSPPOVTLFSP [7]	- Soluble monomer (G_1^{na}) [8]	- R transcripts in <i>Torpedo</i> electric organ [4], in mouse embryonic muscle [9]; expression level increased by stress in mouse brain [10]
H	<i>Drosophila</i> : GT@DGDSS@SASISPRQLQLGIAALYICAAIRTKRVE [11] <i>Torpedo</i> AChE: A@DGLS@SSTSSSKGIIFYVLESILYLIEX [4,12] Rat AChE: ATEVP@T@PSPAHO@EAPREGPALSLLEFLFLHSLRHL [5,13]	- Amphiphilic, intracellular precursors (G_2^a) - GPI-anchored dimers, exposed at the cell surface (G_2^a , type I) - Soluble, secreted C-terminal cleaved dimers (G_2^{na})	- nervous tissue in insects [14] - muscles, nervous tissue and electric organs in <i>Torpedo</i> [4,15] - hematopoietic cells in mammals [8]
T	<i>C. elegans</i> ACE-1: ADVGDPYLVMKQMDKQWNEYITDQYHFEQYKRYQTYRQSDSET@GG [16] <i>Torpedo</i> AChE: ETIDEAERQWKEFHRW-SSYHGHKWKQFDHY-----SRHEN@AEL [17,18] <i>Electrophorus</i> AChE: ENIDDAERQWKAEPHRW-SSYHGHKWKQFDHY-----SKQER@TNL [19] <i>Bungarus</i> AChE: DNIEBAERQWKEFHRW-SAYHGHKWKQFDHY-----NKQDR@SEL [20] Quail AChE: GPTDAER-WRLDFHRW-SSYHGHKWKQFDHY-----SRQQR@ATL [21] Rat AChE: DTLDEAERQWKAEPHRW-SSYHGHKWKQFDHY-----SKQDR@SDL [22] Human AChE: GNIDEAEWKAEPHRW-NFYHGHKWKQFDHY-----SKKES@VGL [23,24]	- Amphiphilic monomers, dimers and tetramers (G_1^a , G_2^a , G_4^a , type II), cellular and secreted [25,26] - Truncated nonamphiphilic forms (G_1^{na}) [27] - Nonamphiphilic tetramers (G_4^{na}) [26] - Heteromeric associations with anchoring subunits: □ collagen ColQ (collagen-tailed, asymmetric forms, A4, A8, A12) [28,29] □ 20 kDa hydrophobic anchor (G_4^a) [13,30-33]	- muscles and nervous tissues in all vertebrates [3]
S	<i>Bungarus</i> AChE: VDFPRADRRERSARA [20,34]	- Soluble monomer (G_1^{na}) [34]	- venom glands and other tissues in Elapid snakes (<i>Bungarus</i>) [35]

Figure 1. Different types of C-terminal peptides in cholinesterases. The underlined regions are removed from the mature proteins; the last residue of the mature protein (ω) is shown in bold type, and the C-terminal region is shown in italics. A C-terminal part of the T peptide may be removed upon secretion, yielding amphiphilic or non-amphiphilic forms, but the sites of cleavage have not been determined. Conserved aromatic residues in T peptides are shown in bold letters; in H and T peptides, cysteines that may establish intercatenary disulfide bonds are shaded. For additional sequences, see the ESTHER server [7].

produce other types of subunits, by alternative splicing of the 3' coding exon. *Torpedo* and mammals possess AChEs of types H, T and possibly R, although R subunits have not been characterized in vivo. Elapid snakes, that secrete AChE in their venom, possess AChEs of types T and S [20]. Only AChE of type T seems to exist in bony fishes (*Danio*, *Electrophorus*) [19, 40] and birds (chicken, quail) (Anselmet, unpublished result). In *Torpedo* and mammals, exons H are located between the last catalytic exon and exon T [4, 6]. Examination of the corresponding sequences in the teleost fish *Electrophorus* [19], in the snake *Bungarus* [20] and in the chicken did not reveal the presence of any potential region that might constitute an exon H encoding a C-terminal peptide with the required features.

In fact, the sequences of H exons in *Torpedo* and mammals do not appear homologous, and only resemble each other by the presence of a cysteine and a GPI addition signal, which is extremely flexible in its amino acid sequence (figure 1). Since mammals are descended from reptiles and teleost fishes, it seems that during evolution of the vertebrate lineages, the exon H as it exists in *Torpedo* was lost, and that a new exon H appeared de novo in mammals.

The C-terminal domains determine the post-translational processing and the fate of the enzyme. Accordingly, when different types of subunits exist in an organism, they are expressed in distinct territo-

ries, in a tissue- and cell-specific manner. In *Torpedo*, AChE_H and AChE_T subunits coexist in the electric organ: AChE_H is produced in the electric motoneurons, and also post-synaptically together with AChE_T. In contrast, the striated muscle of *Torpedo* only seems to contain AChE_H subunits (Bon, unpublished result). In the elapid snake *Bungarus*, AChE_S is expressed in the venom gland, but also in the liver and in muscles, together with AChE_T [20]. In adult mammals, AChE_H is mostly expressed in blood cells (lymphocytes and erythrocytes), while AChE_T is mainly expressed in muscles and in the central and peripheral nervous tissues [5, 22, 41]. In mammalian muscle, however, an exclusive splicing of AChE transcripts towards T exons is only established progressively: in the mouse embryonic diaphragm, R and H transcripts coexist with T transcripts and disappear completely at birth [9].

Thus, the tissue expression of the different types of AChE subunits differs considerably, according to the species. *Drosophila* uses AChE_H in its nervous system, *Torpedo* uses AChE_H in its muscles, while the corresponding tissues in adult mammals only contain AChE_T.

3. Post-translational processing

In this section, we will discuss the manner in which the different C-terminal domains of cholinesterases affect their post-translational fate. It is im-

portant to note that these C-terminal domains are not required for catalytic activity. Truncated subunits (AChE_D), which are essentially reduced to their catalytic domain, may be constructed by introducing a stop codon either at the end of the catalytic domain, or after the first few residues of one of the C-terminal peptides. Such subunits produce active AChE when expressed in transfected cells, in the form of soluble monomers that are readily secreted into the medium [42]. If a cysteine is present after the catalytic domain, such subunits are able to produce disulfide-linked dimers. This is the case if a stop codon is introduced after the cysteine(s) of an H peptide, or in abnormally spliced subunits obtained in the case of *Electrophorus* AChE [19]: when the T exon is deleted or when its acceptor site is mutated, the last catalytic exon is spliced to upstream sites, located 6 bp apart from each other, which introduce very short coding sequences containing two adjoining cysteines, VLECC and ECC.

We will examine the various types of natural subunits, in the order of increasing complexity, which is also the order of increasing physiological importance in higher vertebrates.

3.1. R subunits

R transcripts have been found in *Torpedo*, mouse and rat [4, 5, 8], whose AChE genes contain two alternative C-terminal exons. It is therefore possible that they represent a default product and do not produce a physiologically significant version of AChE. The actual protein has not been identified in tissues, but this is not conclusive. It is intriguing that the R transcripts are specifically up-regulated in the mouse brain after exposure to anticholinesterases or to stress [10]. When synthesized in transfected cells, the mouse R subunits form soluble monomers [8], in agreement with the fact that their C-terminal domain (R peptide) contains neither a cysteine that might form intercatenary disulfide bonds, nor an hydrophobic sequence that might constitute a membrane anchor.

3.2. S subunits

The S exon has recently been discovered in the *Bungarus* AChE gene, and is alternatively spliced with a T exon [20]. The S exon is expressed in the venom glands, and produces a soluble monomeric form of AChE. It encodes a short C-terminal peptide of 15 residues, which is very hydrophilic and does not contain any cysteine. Curiously, this peptide is cleaved upon secretion so that the mature venom protein retains only its first seven amino acids. The secreted enzyme produced by transfected COS cells is also cleaved, as indicated by the loss of a flag epitope that was introduced at the C-terminus by ge-

netic construction in expression vectors. This cleavage does not seem necessary for an efficient expression and secretion of the enzyme, since we observed a similar yield in the case of a truncated form, in which a stop codon was introduced at the end of the catalytic domain.

3.3. H subunits

Subunits of type H are characterized by the fact that their C-terminal peptide contains one or two cysteines near the catalytic domain, and a signal for addition of a glycoposphatidylinositol anchor (GPI). The AChE_H subunits thus generate disulfide-linked, GPI-anchored dimers. Since these dimers are globular and amphiphilic, we note them G₂^a; they correspond to amphiphilic forms of type I, as opposed to those produced from subunits of type T.

The GPI addition signal consists of a cleavage/addition site, located about 10–12 residues upstream of a C-terminal hydrophobic region. At the cleavage/addition site, a peptide bond is replaced by an amide bond to the ethanolamine moiety of a preassembled GPI anchor. This anchor thus becomes attached to the last remaining residue of the mature protein, which is called ω . Analyses of GPI addition signals and site-directed mutagenesis studies have shown that ω must possess a small side chain, and that a similar restriction may also apply to the adjoining residues, $\omega + 1$ and $\omega + 2$ [43, 44].

We have analyzed the environment of ω sites in *Torpedo* and rat H peptides by mutagenesis and this led us to reconsider some of the previously proposed consensus rules (Bon et al., in preparation). For example, we found that the ω -1 residue has a strong influence on processing. It is particularly interesting that the production of GPI-anchored AChE, which becomes exposed at the cell surface, is accompanied by release of a soluble form in the culture medium. The efficiency of processing, i.e., the ratio of GPI-anchored molecules to uncleaved precursors, which remain trapped in vesicles within the cells, appeared generally correlated with the release of soluble enzyme, and with the total yield of AChE activity. This yield varied more than 10-fold from unprocessed or poorly processed mutants to the better processed ones.

In these analyses, we used an operational distinction between amphiphilic (G^a) and non-amphiphilic (G^{na}) molecules: amphiphilic molecules are defined by their capacity to bind micelles of non-denaturing detergents, such as Triton X-100 and Brij-96, resulting in clear changes in their hydrodynamic parameters. The sedimentation of amphiphilic molecules is retarded in the presence of Triton X-100, and even more so in the presence of Brij-96; the Stokes radius

is increased and the rate of migration of amphiphilic molecules in non-denaturing polyacrylamide gels is decreased in the presence of Triton X-100. According to these criteria, the GPI-anchored forms and the precursors which had retained their C-terminal hydrophobic region are amphiphilic, while the released enzyme is nonamphiphilic. This released form may constitute a large fraction of the enzyme produced from AChE_H, and thus it may be physiologically significant, e.g., by contributing to the soluble AChE activity that exists in the sera of some mammalian species.

GPI-anchored AChE is clearly essential in invertebrates where it is the only or predominant cholinesterase in the nervous system, and also appears to serve an essential role in *Torpedo*, since this is the only form of enzyme in the striated muscles. In mammals, the expression of AChE_H in hematopoietic cells may be related to their differentiation [45, 46]. However, the physiological significance of GPI-anchored AChE on the surface of blood cells is not clear, especially since its activity varies widely with the species (and does not exist in reptiles and birds). In fact, the presence of AChE on red blood cells may be redundant with that of soluble AChE and BChE in the plasma. It would be interesting to specifically inactivate the exon H in mouse, in order to observe whether the absence of GPI-anchored molecules would induce any functional defect.

3.4. T subunits

As already indicated, subunits of type T seem to exist in all vertebrates AChEs. Vertebrate BChEs and the product of the major *ace-1* gene in *C. elegans* also belong to this category. The characteristic C-terminal regions of these subunits, the T peptides, are 40 residues long in vertebrate AChEs, and remarkably well conserved throughout evolution, with a series of aromatic residues and a cysteine located near the C-terminus (at position -4 in vertebrate AChEs and BChEs) (figure 1).

The first part of the T peptide probably organizes as an amphiphilic α -helix, with all aromatic side chains forming an hydrophobic patch [3], as shown by spectroscopic analyses of a synthetic peptide, in the presence of detergent and lipid micelles (Bon et al., in preparation). In a proposed theoretical model, the α -helix bends upon itself, creating an hydrophobic zone [47]. This type of conformation probably explains the multiple interactions of cholinesterase subunits of type T. These subunits generate various oligomeric forms, ranging from monomers to the complex heteromeric collagen-tailed and hydrophobic-tailed molecules, in which they are associated with structural proteins.

Monomers and dimers occur in tissues and in transfected cells that express AChE_T subunits, in variable proportions depending on the species. For example, dimers are predominant in the case of *Torpedo* AChE_T [42], whereas monomers are predominant in the case of rat AChE_T [22, 26]. These molecules are amphiphilic, but differ from GPI-anchored forms because they are more easily solubilized in an aqueous buffer without detergent, and do not form aggregates. They were thus called amphiphilic forms of type II, as opposed to GPI-anchored amphiphilic forms of type I [48–50]. Transfected COS cells expressing AChE_T secrete amphiphilic monomers (G₁^a) and dimers (G₂^a), which resemble their cellular counterparts but differ in their electrophoretic migration in non-denaturing polyacrylamide gels, and probably undergo a proteolytic cleavage at their C-terminus, as shown by the loss of a flag peptide epitope, which was added by mutagenesis at the end of some constructs [26].

In cultures of murine neural cells, it was shown that monomers and dimers are rapidly renewed, presenting several metabolic components with short half-lives (less than 8 h), while the half life of tetramers exceeds 20 h [51].

In addition to monomers and dimers, transfected COS cells, expressing AChE_T subunits, also produce tetramers and heavier oligomers [26]. The tetramers exist as both amphiphilic and nonamphiphilic molecules, which possibly differ by the organization of their T peptides: in amphiphilic molecules, at least part of the hydrophobic region of the T peptide would remain exposed, while they would be occluded within an internal hydrophobic core in the non-amphiphilic molecules. These interpretations assume that such tetramers only consist of AChE_T subunits; there is, however, another possibility, as discussed below.

The transfected cells also produce a '13 S' component, which might correspond to an hexamer of AChE_T subunits. This type of molecule has been observed in COS cells, in *Xenopus* oocytes (Simon et al., in preparation), and also in primary cultures of murine nerve cells (Lazar, unpublished result). It is not amphiphilic and may be secreted, e.g., from transfected oocytes. It is quite unstable, being readily dissociated into tetramers, dimers and monomers, even around room temperature. The proportion of resulting amphiphilic tetramers is increased when dissociation occurs in the presence of detergent, suggesting that interaction with micelles favors a conformation in which the amphiphilic α -helix is exposed.

The presence of the T peptide also endows AChE_T subunits to form heteromeric associations. In the collagen-tailed or asymmetric (A) forms, each strand

of a triple helical collagen may be attached to a tetramer of AChE_T subunits: two of these subunits are disulfide-linked to each other, while the other two are disulfide-linked to the tail subunit. In the A₁₂ form, each of the three strands of the collagen is attached to a catalytic tetramer. When one or two of these strands remain unoccupied, the corresponding molecular forms are called A₈ and A₄, indicating their respective number of catalytic subunits. The hydrophobic-tailed, membrane-bound tetramers, which represent the major AChE species in mammalian brain, contain an hydrophobic protein of 20 kDa [30, 31] and present the same type of disulfide-bond organization [13].

4. Heteromeric assembly between T subunits and the collagen tail, ColQ

The AChE-associated collagen of asymmetric forms has been cloned in *Torpedo* [28] and in mammals [29]. The collagen subunit, ColQ, consists of a signal peptide, an N-terminal region, Q_N, a collagen central domain that is bracketed by cysteines, and a C-terminal region, Q_C, containing proline-rich and cysteine-rich conserved motifs.

In mammals, we found a single ColQ gene, and showed that ColQ subunits form homomeric triple helices that constitute the collagen tail in asymmetric forms of both AChE and BChE. The ColQ gene generates a number of splicing variants, some of which do not encode the Q_N or the Q_C regions. Their significance has not yet been elucidated. The Q_N region is responsible for the binding of AChE_T tetramers. We could, for example, generate soluble tetramers by co-expressing AChE_T with a Q_N construct, without the collagen and Q_C regions, or GPI-anchored tetramers with a Q_N/H_C construct, in which Q_N was combined with the C-terminal signal for addition of a GPI anchor, as encoded by an H exon. The Q_N domains of *Torpedo* and rat are able to associate with T subunits from various origins, indicating a strong conservation of their complementarity through evolution.

Comparison of the Q_N sequences showed a conserved motif of 17 residues, that includes two adjoining cysteines and strings of five and three consecutive prolines. Deletion studies showed that this domain is sufficient for interaction with cholinesterase subunits of type T, and we called it a proline-rich attachment domain, PRAD [52]. Mutation studies further showed that the cysteines were dispensable, so that disulfide bonding between the catalytic subunits and the tail is not necessary, and that the only critical element of the PRAD is the presence of a sufficient number of successive prolines. We could in fact replace the PRAD by synthetic poly-

proline, inducing the recruitment of monomers and dimers of AChE_T into tetramers, both in cultures of living cells, and in cell extracts.

Having established that the complementation between catalytic subunits and tail subunits relies on the small PRAD motif of the tail, we wondered whether, similarly, the T peptide might be sufficient for this interaction (Simon et al., in preparation). This appeared possible, because: i) the T peptide is required for this interaction and contains the cysteine that may form disulfide bonds between the two partners; ii) it is very well conserved throughout vertebrates, markedly more than the catalytic domains, since the catalytic domains of *Torpedo* and rat AChE present 59% identity, whereas their T peptides present 75% identity; and iii) the small size of the PRAD suggests that it interacts with an appropriately small domain.

We demonstrated that, indeed, an isolated T peptide, which was labeled with a flag epitope, could associate with the Q_N/H_C protein and was thus GPI-anchored at the cell membrane of transfected COS cells. We further showed that addition of a T peptide at the C-terminus of foreign proteins such as alkaline phosphatase and the green fluorescent protein, GFP, endowed them with the capacity to associate with the PRAD, when the two partners were synthesized in the secretory pathway.

Sedimentation analyses showed that mixed oligomers were formed in *Xenopus* oocytes, when the PRAD was expressed together with AChE_T subunits and isolated T peptides or the GFP-T protein. The mixed oligomers contained three, two, or one AChE_T subunits, showing that a PRAD combines with four T peptides. We observed, however, a bias in favor of the 2/2 combination, suggesting that the PRAD associates sequentially with dimers of T peptides, probably forming unstable intermediates before the stabilization of the tetrameric assembly. The replacement of the C-terminal cysteine by an alanine did not affect these interactions, showing that disulfide bonds between T peptides dimers are not required, any more than disulfide bonds between the PRAD and the T peptides. The formation of mixed 2/2 oligomers certainly explains the existence of AChE/BChE asymmetric forms, which were characterized in chick embryos [53].

The interaction between the PRAD and T peptides superficially resembles the binding of proline-rich ligands to SH3 and WW domains [54]. It is, however, quite original in several respects. Firstly, it does not occur in the cytoplasm, but in the secretory pathway, between proteins that are destined to be externalized. Secondly, it presents a one to four stoichiometry. In the case of the trimeric ColQ collagen, this leads to the A₁₂ collagen-tailed molecule, combining three

structural subunits and 12 catalytic subunits. This type of association clearly offers the potential to generate complex supramolecular structures.

5. Generality of the PRAD/T peptide interaction

As mentioned above, the AChE_T subunits associate with a 20 kDa hydrophobic membrane anchor [13, 30–33], called P [55], with the same type of disulfide bond organization as with the PRAD [13]. This suggested that the hydrophobic P subunit might be encoded by a splice variant generated from the ColQ gene, and contained the PRAD. However, this possibility was not confirmed by an examination of the splice variants produced in mammalian brain, and the fact that membrane-bound AChE tetramers do not contain a peptide epitope which is encoded by the same exon as the PRAD [29]. It is therefore likely that the P subunit is produced by a distinct gene, and it will be interesting to see whether it also contains a proline-rich domain. In any case, this indicates the existence of several peptide motifs that may associate with T subunits and induce the formation of AChE_T tetramers.

An intriguing hypothesis is that AChE_T subunits might never be able to form tetramers by themselves, in the absence of such an 'organizer' molecule. This would explain the fact that tetramers are clearly not in equilibrium with dimers and monomers and that their proportion varies widely with differentiation, in vivo, or according to the cell type in transfected cell cultures. For example, the ratio of tetramers to monomers and dimers increases considerably during maturation of the brain, in birds [21] and mammals [41].

Transfected COS cells produce a variable, but significant level of G₄^a and G₄^{na} forms, while *Xenopus* oocytes produce essentially no tetramers. These differences would be explained perfectly if the formation of tetramers depends on the expression of organizer proteins. The presence of such a protein has not been detected in cholinesterase tetramers, apart from the membrane-bound tetramers of mammalian brain. This may be due to its small size, to the fact that it is not necessarily disulfide-linked to catalytic subunits and to its stoichiometry of only one to four catalytic subunits.

In view of the possible existence of multiple, perhaps ubiquitous, PRAD-like motifs, we may also imagine the existence of T peptides in association with proteins that are unrelated to cholinesterases. A possible example is the 100 kDa protein that was found to be a component of the AChE collagen-tailed forms in *Torpedo* electric organs. Thus, the PRAD/T peptide association may be the prototype of a family of similar protein-protein associations,

organizing supramolecular structures in the extracellular space.

6. Physiological importance of AChE anchoring

In any case, associations of AChE catalytic subunits with structural proteins, the collagen tail ColQ and the hydrophobic membrane anchor P, play a major role in the positioning and function of the enzyme in cholinergic transmission. This is perfectly illustrated by a congenital myasthenic syndrome (CMS of type 1c), which is characterized by a defect in the accumulation of AChE at neuromuscular junctions. This syndrome is usually correlated with a complete or partial lack of collagen-tailed forms [56]. An analysis of the AChE gene of a CMS-1c patient showed that it presented no anomaly and produced AChE_T subunits that normally associated with a PRAD in transfected cells [57]. We therefore proposed that the genetic defect might reside in the ColQ gene. This was confirmed in a recent genetic study of a large kindred in which several siblings are affected by a mild form of CMS-1c (Donger et al., in preparation). It is particularly interesting that muscle biopsies from these patients contain a normal complement of collagen-tailed AChE, although this enzyme is not properly focalized at the neuromuscular junctions. (Note that human and primate muscles contain an important proportion of collagen-tailed AChE in extrajunctional regions of muscles, in contrast with the rat.) In these patients, we identified a point mutation which changes a conserved tyrosine residue in the C-terminal Q_C region of ColQ, and we suggest that this mutation compromises the interaction of Q_C with specific elements of the junctional basal lamina. This would be consistent with the 'parking lot' hypothesis, according to which collagen-tailed molecules attach to a limiting number of 'receptor' sites in the architecture of the neuromuscular junction [58], possibly in connection with the receptor sites.

The collagen-tailed forms, however, do not constitute the only type of physiologically active AChE species in muscles: the studies of Gisiger et al. [59] have shown that exercise induces specific changes in the membrane-bound G₄ form of rat muscles, but not in the other molecular forms of AChE, strongly suggesting that the level of G₄ has an adaptive role, related to muscle activity.

These examples show the importance of the stoichiometric and spatial organization of AChE catalytic sites and acetylcholine receptor sites for cholinergic function. Although AChE is produced from a single gene, its various molecular forms, generated by alternative splicing and by combinations with structural proteins, allow multiple modes of anchoring. This multiplicity is probably the functional counterpart of the

various types of nicotinic and muscarinic receptors, which differ widely in their affinity for acetylcholine and time course of response.

Acknowledgments

This work was supported by the Centre National de la Recherche Scientifique, the Direction des Systèmes de Forces et de la Prospective, the Association Française contre les Myopathies, and the European Community. We thank Annick Ayon, Monique Lambergeon, Anne Le Goff and Jacqueline Leroy for expert technical assistance.

References

- [1] Sussman J.L., Harel M., Frolow F., Oefner C., Goldman A., Tokar L., Silman I., Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein, *Science* 253 (1991) 872–879.
- [2] Ollis D.L., Cheah E., Cygler M., Dijkstra B., Frolow F., Franken S.M., Harel M., Remington S.J., Silman I., Schrag J. et al., The alpha/beta hydrolase fold, *Protein Eng.* 5 (1992) 197–211.
- [3] Massoulié J., Pezzementi L., Bon S., Krejci E., Vallette F.M., Molecular and cellular biology of cholinesterases, *Prog. Neurosci.* 41 (1993) 31–91.
- [4] Sikorav J.L., Duval N., Anselmet A., Bon S., Krejci E., Legay C., Osterlund M., Reimund B., Massoulié J., Complex alternative splicing of acetylcholinesterase transcripts in *Torpedo* electric organ; primary structure of the precursor of the glycolipid-anchored dimeric form, *EMBO J.* 7 (1988) 2983–2993.
- [5] Legay C., Bon S., Massoulié J., Expression of a cDNA encoding the glycolipid-anchored form of rat acetylcholinesterase, *FEBS Lett.* 315 (1993) 163–166.
- [6] Li Y., Camp S., Rachinsky T.L., Getman D., Taylor P., Gene structure of mammalian acetylcholinesterase. Alternative exons dictate tissue-specific expression, *J. Biol. Chem.* 266 (1991) 23083–23090.
- [7] Cousin X., Hotelier T., Liévin P., Toutant J.P., Chatonnet A., A cholinesterase genes server (ESTHER): a database of cholinesterase-related sequences for multiple alignments, phylogenetic relationships, mutations and structural data retrieval, *Nucleic Acids Res.* 24 (1996) 132–136.
- [8] Li Y., Camp S., Taylor P., Tissue-specific expression and alternative mRNA processing of the mammalian acetylcholinesterase gene, *J. Biol. Chem.* 268 (1993) 5790–5797.
- [9] Legay C., Huchet M., Massoulié J., Changeux J.P., Developmental regulation of acetylcholinesterase transcripts in the mouse diaphragm: alternative splicing and focalization, *Eur. J. Neurosci.* 7 (1995) 1803–1809.
- [10] Kaufer D., Friedman A., Seidman S., Soreq H., Acute stress facilitates long-lasting changes in cholinergic gene expression, *Nature* 393 (1998) 373–377.
- [11] Fournier D., Karch F., Bride J.-M., Hall L.M.C., Bergé J.-B., Spierer P., *Drosophila melanogaster* acetylcholinesterase gene: structure, evolution and mutations, *J. Mol. Biol.* 210 (1990) 15–22.
- [12] Bucht G., Hjalmarsson K., Residues in *Torpedo californica* acetylcholinesterase necessary for processing to a glycosyl phosphatidylinositol-anchored form, *Biochim. Biophys. Acta* 1292 (1996) 223–232.
- [13] Roberts W.L., Doctor B.P., Foster J.D., Rosenberry T.L., Bovine brain acetylcholinesterase primary sequence involved in intersubunit disulfide linkages, *J. Biol. Chem.* 266 (1991) 7481–7487.
- [14] Toutant J.-P., Insect acetylcholinesterase: catalytic properties, tissue distribution and molecular forms, *Prog. Neurobiol.* 32 (1988) 423–446.
- [15] Schumacher M., Maulet Y., Camp S., Taylor P., Multiple messenger RNA species give rise to the structural diversity in acetylcholinesterase, *J. Biol. Chem.* 263 (1988) 18979–18987.
- [16] Arpagaus M., Fedon Y., Cousin X., Chatonnet A., Bergé J.-B., Fournier D., Toutant J.-P., cDNA sequence, gene structure, and in vitro expression of *ace-1*, the gene encoding acetylcholinesterase of class A in the nematode *Caenorhabditis elegans*, *J. Biol. Chem.* 269 (1994) 9957–9965.
- [17] Schumacher M., Camp S., Maulet Y., Newton M., MacPhee-Quigley K., Taylor S.S., Friedmann T., Taylor P., Primary structure of *Torpedo californica* acetylcholinesterase deduced from its cDNA sequence, *Nature* 319 (1986) 407–409.
- [18] Sikorav J.L., Krejci E., Massoulié J., cDNA sequences of *Torpedo marmorata* acetylcholinesterase: primary structure of the precursor of a catalytic subunit; existence of multiple 5'-untranslated regions, *EMBO J.* 6 (1987) 1865–1873.
- [19] Simon S., Massoulié J., Cloning and expression of acetylcholinesterase from *Electrophorus*: splicing pattern of the 3' exons in vivo and in transfected mammalian cells, *J. Biol. Chem.* 273 (1997) 33045–33055.
- [20] Cousin X., Bon S., Massoulié J., Bon C., Identification of a novel type of alternatively spliced exon from the acetylcholinesterase gene of *Bungarus fasciatus*. Molecular forms of acetylcholinesterase in the snake liver and muscle, *J. Biol. Chem.* 273 (1998) 9812–9820.
- [21] Anselmet A., Fauquet M., Chatel J.M., Maulet Y., Massoulié J., Vallette F.M., Evolution of acetylcholinesterase transcripts and molecular forms during development in the central nervous system of the quail, *J. Neurochem.* 62 (1994) 2158–2165.
- [22] Legay C., Bon S., Vernier P., Coussen F., Massoulié J., Cloning and expression of a rat acetylcholinesterase subunit: generation of multiple molecular forms and complementarity with a *Torpedo* collagenic subunit, *J. Neurochem.* 60 (1993) 337–346.
- [23] Lockridge O., Bartels C.F., Vaughan T.A., Wong C.K., Norton S.E., Johnson L.L., Complete amino acid sequence of human serum cholinesterase, *J. Biol. Chem.* 262 (1987) 549–557.
- [24] Arpagaus M., Kott M., Vatsis K.P., Bartels C.F., La Du B.N., Lockridge O., Structure of the gene for human butyrylcholinesterase. Evidence for a single copy, *Biochemistry* 29 (1990) 124–131.
- [25] Bon S., Rosenberry T.L., Massoulié J., Amphiphilic, glycosylphosphatidylinositol-specific phospholipase C (PI-PLC)-insensitive monomers and dimers of acetylcholinesterase, *Cell. Mol. Neurobiol.* 11 (1991) 157–172.
- [26] Bon S., Massoulié J., Quaternary associations of acetylcholinesterase. I Oligomeric associations of T subunits with and without the amino-terminal domain of the collagen tail, *J. Biol. Chem.* 272 (1997) 3007–3015.
- [27] Liao J., Boschetti N., Mortensen V., Jensen S.P., Koch C., Norgaard-Pedersen B., Brodbeck U., Characterization of salt-soluble forms of acetylcholinesterase from bovine brain, *J. Neurochem.* 63 (1994) 1446–1453.
- [28] Krejci E., Coussen F., Duval N., Chatel J.M., Legay C., Puype M., Vandekerckhove J., Cartaud J., Bon S., Massoulié J., Primary structure of a collagenic tail peptide of *Torpedo* acetylcholinesterase: co-expression with catalytic subunit indu-

- ces the production of collagen-tailed forms in transfected cells, *EMBO J.* 10 (1991) 1285–1293.
- [29] Krejci E., Thomine S., Boschetti N., Legay C., Sketelj J., Massoulié J., The mammalian gene of acetylcholinesterase-associated collagen. *J. Biol. Chem.* 272 (1997) 22840–22847.
 - [30] Gennari K., Brunner J., Brodbeck U., Tetrameric detergent-soluble acetylcholinesterase from human caudate nucleus: subunit composition and number of active sites, *J. Neurochem.* 49 (1987) 12–18.
 - [31] Inestrosa N.C., Roberts W.L., Marshall T.L., Rosenberry T.L., Acetylcholinesterase from bovine caudate nucleus is attached to membranes by a novel subunit distinct from those of acetylcholinesterases in other tissues, *J. Biol. Chem.* 262 (1987) 4441–4444.
 - [32] Boschetti N., Liao J., Brodbeck U., The membrane form of acetylcholinesterase from rat brain contains a 20 kDa hydrophobic anchor, *Neurochem. Res.* 19 (1994) 359–365.
 - [33] Boschetti N., Brodbeck U., The membrane anchor of mammalian brain acetylcholinesterase consists of a single glycosylated protein of 22 kDa, *FEBS Lett.* 380 (1996) 133–136.
 - [34] Cousin X., Bon S., Duval N., Massoulié J., Bon C., Cloning and expression of acetylcholinesterase from *Bungarus fasciatus* venom. A new type of COOH-terminal domain; involvement of a positively charged residue in the peripheral site, *J. Biol. Chem.* 271 (1996) 15099–15108.
 - [35] Cousin X., Créminon C., Grassi J., Méflah K., Cornu G., Saliou B., Bon S., Massoulié J., Bon C., Acetylcholinesterase from *Bungarus venom*: a monomeric species, *FEBS Lett.* 387 (1996) 196–200.
 - [36] Bourguet D., Raymond M., Fournier D., Malcolm C.A., Toutant J.-P., Arpagaus M., Existence of two acetylcholinesterase in the mosquito *Culex pipiens* (Diptera: Culicidae), *J. Neurochem.* 67 (1996) 2115–2123.
 - [37] Sutherland D., McClellan D.S., Milner D., Soong W., Axon N., Sanders M., Hester A., Kao Y.-H., Poczek T., Routt S., Pezzementi L., Two cholinesterase activities and genes are present in amphioxus, *J. Exp. Zool.* 277 (1997) 213–229.
 - [38] Grauso M., Culetto E., Combes D., Fedon Y., Toutant J.-P., Arpagaus M., Existence of four acetylcholinesterase genes in the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*, *FEBS Lett.* 424 (1998) 279–284.
 - [39] Harel M., Sussman J.L., Krejci E., Bon S., Chanal P., Massoulié J., Silman I., Conversion of acetylcholinesterase to butyrylcholinesterase: modeling and mutagenesis, *Proc. Natl. Acad. Sci. USA* 89 (1992) 10827–10831.
 - [40] Bertrand C., Takke C., Cousin X., Toutant J.P., Chatonnet A., in: *Zebrafish development and genetics*, Cold Spring Harbor, 1996, 105 p.
 - [41] Toutant J.P., Massoulié J., Cholinesterases: tissue and cellular distribution of molecular forms and their physiological regulation, *Handb. Exp. Pharmacol.* 86 (1988) 225–265.
 - [42] Duval N., Massoulié J., Bon S., H and T subunits of acetylcholinesterase from *Torpedo*, expressed in COS cells, generate all types of globular forms, *J. Cell Biol.* 118 (1992) 641–653.
 - [43] Kodukula K., Gerber L.D., Amthauer R., Brink L., Udenfriend S., Biosynthesis of glycosylphosphatidylinositol (GPI) anchored membrane proteins in intact cells: specific amino acid requirements adjacent to the site of cleavage and GPI attachment, *J. Cell Biol.* 120 (1993) 657–664.
 - [44] Moran P., Caras I.W., Requirements for glycosylphosphatidylinositol attachment are similar but not identical in mammalian cells and parasitic protozoa, *J. Cell Biol.* 125 (1994) 333–343.
 - [45] Lapidot-Lifson Y., Prody C.A., Ginzberg D., Meytes D., Zakut H., Soreq H., Coamplification of human acetylcholinesterase and butyrylcholinesterase genes in blood cells: correlation with various leukemias and abnormal megakaryocytopoiesis, *Proc. Natl. Acad. Sci. USA* 86 (1989) 4715–4719.
 - [46] Patinkin D., Seidman S., Eckstein F., Benseler F., Zakut H., Soreq H., Manipulations of cholinesterase gene expression modulate murine megakaryocytopoiesis in vitro, *Mol. Cell. Biol.* 10 (1990) 6046–6050.
 - [47] Giles K., Interactions underlying subunit association in cholinesterases, *Protein Eng.* 10 (1997) 677–685.
 - [48] Bon S., Toutant J.P., Méflah K., Massoulié J., Amphiphilic and nonamphiphilic forms of *Torpedo* cholinesterases: I. Solubility and aggregation properties, *J. Neurochem.* 51 (1988) 776–785.
 - [49] Bon S., Toutant J.P., Méflah K., Massoulié J., Amphiphilic and nonamphiphilic forms of *Torpedo* cholinesterases: II. Electrophoretic variants and phosphatidylinositol phospholipase C-sensitive and -insensitive forms, *J. Neurochem.* 51 (1988) 786–794.
 - [50] Massoulié J., Sussman J., Bon S., Silman I., Structure and functions of acetylcholinesterase and butyrylcholinesterase, *Prog. Brain Res.* 98 (1993) 139–146.
 - [51] Lazar M., Salmeron E., Vigny M., Massoulié J., Heavy isotope-labeling study of the metabolism of monomeric and tetrameric acetylcholinesterase forms in the murine neuronal-like T 28 hybrid cell line, *J. Biol. Chem.* 259 (1984) 3703–3713.
 - [52] Bon S., Coussen F., Massoulié J., Quaternary associations of acetylcholinesterase; II. the polyproline attachment domain of the collagen tail, *J. Biol. Chem.* 272 (1997) 3016–3021.
 - [53] Tsim K.W., Randall W.R., Barnard E.A., An asymmetric form of muscle acetylcholinesterase contains three subunit types and two enzymic activities in one molecule, *Proc. Natl. Acad. Sci. USA* 85 (1988) 1262–1266.
 - [54] Chan D.C., Bedford M.T., Leder P., Formin binding proteins bear WWP/WW domains that bind proline-rich peptides and functionally resemble SH3 domains, *EMBO J.* 15 (1996) 1045–1054.
 - [55] Massoulié J., Sussman J.L., Doctor B.P., Soreq H., Velan B., Cygler M., Rotundo R.L., Shafferman A., Silman I., Taylor P., in: Shafferman A., Velan B. (Eds.), *Multidisciplinary approaches to cholinesterase functions*, Plenum Press, New York, 1992, pp. 285–288.
 - [56] Hutchinson D.O., Engel A.G., Walls T.J., Nakano S., Camp S., Taylor P., Harper C.M., Brengman J.M., The spectrum of congenital end-plate acetylcholinesterase deficiency, *Ann. N.Y. Acad. Sci.* 21 (1993) 469–486.
 - [57] Camp S., Bon S., Li Y., Getman D.K., Engel A.G., Massoulié J., Taylor P., Patients with congenital myasthenia associated with end-plate acetylcholinesterase deficiency show normal sequence, mRNA splicing, and assembly of catalytic subunits, *J. Clin. Invest.* 95 (1995) 333–340.
 - [58] Rossi S.G., Rotundo R.L., Transient interactions between collagen-tailed acetylcholinesterase and sulfated proteoglycans prior to immobilization on the extracellular matrix, *J. Biol. Chem.* 271 (1996) 1979–1987.
 - [59] Gisiger V., Bélisle M., Gardiner P.F., Acetylcholinesterase adaptation to voluntary wheel running is proportional to the volume of activity in fast, but not slow, rat hindlimb muscles, *Eur. J. Neurosci.* 6 (1994) 673–680.

Three-dimensional structure of a complex of E2020 with acetylcholinesterase from *Torpedo californica*

Gitay Kryger^a, Israel Silman^b, Joel L. Sussman^{a, c}

Departments of ^aStructural Biology and ^bNeurobiology, Weizmann Institute of Science, Rehovoth 76100, Israel
^cBiology Department, Brookhaven National Laboratory, Upton, NY 11973, USA

Abstract — The 3D structure of a complex of the anti-Alzheimer drug, E2020, also known as Aricept[®], with *Torpedo californica* acetylcholinesterase is reported. The X-ray structure, at 2.5 Å resolution, shows that the elongated E2020 molecule spans the entire length of the active-site gorge of the enzyme. It thus interacts with both the ‘anionic’ subsite, at the bottom of the gorge, and with the peripheral anionic site, near its entrance, via aromatic stacking interactions with conserved aromatic residues. It does not interact directly with either the catalytic triad or with the ‘oxanion hole’. Although E2020 is a chiral molecule, and both the S and R enantiomers have similar affinity for the enzyme, only the R enantiomer is bound within the active-site gorge when the racemate is soaked into the crystal. The selectivity of E2020 for acetylcholinesterase, relative to butyrylcholinesterase, can be ascribed primarily to its interactions with Trp279 and Phe330, which are absent in the latter. (©Elsevier, Paris)

Résumé — Structure d’un complexe de E2020 avec l’acétylcholinestérase de *Torpedo californica*. La structure 3D d’un complexe formé par une drogue anti-Alzheimer E2020 aussi connue sous le nom de Aricept[®] avec l’acétylcholinestérase de *Torpedo californica* est décrite. La structure aux rayons X, à la résolution de 2.5 Å, montre que la molécule allongée E2020 s’étire sur l’entière longueur de la gorge catalytique de l’enzyme. Elle interagit donc à la fois avec le sous-site anionique, au fond de la gorge et avec le site périphérique anionique, près de l’entrée par les interactions avec les résidus conservés aromatiques empilés. (©Elsevier, Paris)

Alzheimer’s disease / drug design / peripheral site

1. Introduction

Acetylcholinesterase (AChE) terminates synaptic transmission at cholinergic synapses by rapid hydrolysis of acetylcholine (ACh) [15]. Anticholinesterase agents are used in the treatment of various disorders [21], and have been proposed as therapeutic agents for the management of Alzheimer’s disease [7]. Two such anticholinesterase agents, both of which act as reversible inhibitors of AChE, have been licensed by the FDA: tacrine (1,2,3,4-tetrahydroacridine) [6], under the trade name Cognex[®], and, more recently, E2020 ((R,S-1-benzyl-4-[5,6-dimethoxy-1-indanon-2-yl]methylpiperidine) [18], under the trade name Aricept[®]. Tacrine and E2020 share the same target, but, whereas tacrine must be administered up to four times a day, and displays hepatotoxic side effects, E2020 may be administered once daily, and has fewer side effects. Furthermore, E2020 displays very high selectivity, ~1000-fold, for AChE relative to butyrylcholinesterase (BChE), whereas THA has similar affinity for the two enzymes. This may be important, since it has been suggested that inhibition of human plasma BChE may cause potentiating side effects [23].

The active site of AChE contains a catalytic subsite, and a so-called ‘anionic’ subsite, which binds the quaternary group of ACh [15]. A second, ‘pe-

ripheral’, anionic site is so named since it is distant from the active site [22]. Bisquaternary inhibitors of AChE derive their enhanced potency, relative to homologous monoquaternary ligands [13], from their ability to span these two ‘anionic’ sites, which are ca. 14 Å apart.

The 3D structure of *Torpedo californica* (Tc) AChE [20] reveals that the active site is located at the bottom of a deep and narrow cavity; named the ‘aromatic gorge’, since > 50% of its lining is composed of the rings of 14 conserved amino acids [1, 20]. The peripheral site is located at the entrance to the gorge [8].

X-ray crystallographic studies of complexes of AChE with drugs of pharmacological interest can reveal which amino acid residues are important for binding the drug, and where space might exist for modifying the drug itself, information crucial for structure-based drug design. In the following, we describe the crystallographic structure, at 2.5 Å resolution, of a complex of E2020 with TcAChE.

2. Materials and methods

2.1. Protein preparation and crystallization

TcAChE was purified and crystallized as described previously [16]. E2020, as the hydrochloride salt of the pure racemate, was a generous gift from Dr. B.P. Doctor (Division of

Biochemistry, Walter Reed Army Institute of Research, Washington, DC, USA). *TcAChE* crystals were soaked in ~10 mM (R,S)E2020 for 5 days at 4 °C, and flash cooled to 100°K.

2.2. X-ray data collection and processing

Data were collected 'inhouse', at the Weizmann Institute of Science, on a Rigaku FR300 generator set at 50 mA, 50 kV and 1.54184 Å wavelength (Cu K radiation), equipped with a R-AXI-SII detector.

2.3. Model refinement and analysis

The structure was refined on the basis of the starting model of native *TcAChE* (PDB ID 2ACE), using only the polypeptide and none of the solvent atoms, employing XPLOR [2]. Voids were calculated between the inhibitor molecule as one entity, and the protein and solvent molecules as a second entity [12].

3. Results and discussion

The 3D structure of the complex shows more detail of the *AChE* structure than the starting native model (2ACE). Primarily, residues 2 and 3, at the N-terminus, and the 484–490 loop, which were not seen in the original model, can be discerned. In addition, it was possible to model the proximal Nacetyl glucosamine (NAG) moiety at four out of five putative glycosylation sites, viz. at residues Asn59, Asn416 (where two NAG moieties could be fitted), Asn457 and Asn533.

3.1. All three segments of E2020 interact with *AChE*

E2020 binds along the active site gorge. All three major segments of the elongated molecule make specific interactions with the enzyme, and each of these interactions involves discrete watermediated contacts which appear crucial for specificity (figure 1). E2020 makes principal interactions with the enzyme through: 1) the benzyl moiety; 2) the piperidine nitrogen; and 3) the dimethoxyindanone moiety. It utilizes the conserved aromatic residues which line the gorge for hydrophobic and stacking interactions, and does not make direct contact with the protein through H-bonds or salt-bridges other than via water molecules.

3.2. Interactions at the bottom of the gorge

Near the bottom of the gorge, one face of the benzyl ring stacks against the six-membered ring of the indole moiety of Trp84, similarly to THA [8]. On the opposite face, it makes a classic aromatic hydrogen bond with a water molecule (WAT1160). This water is held firmly by a hydrogen bond to another water molecule (WAT1161), in the 'oxyanion hole',

and to WAT1159. WAT1161 is another example of a tightly bound water molecule; it hydrogen bonds to residues in the 'oxyanion hole' and to S200O. E2020 does not interact directly either with the catalytic triad or with the 'oxyanion hole'.

3.3. Interactions in the middle of the gorge

In the constricted region, halfway up the gorge, the charged nitrogen of the piperidine ring makes a cation- π interaction [5] with the phenyl ring of Phe330. The ring nitrogen also makes an in-line H-bond with WAT1159. The principal binding site for the quaternary nitrogen of *ACh* within the active site, and for homologous ligands, is the indole ring of Trp84 [9]. These data suggest that Phe330 may serve as an additional quaternary binding site, of possible functional significance, midway down the gorge, between the peripheral binding site and the anionic subsite of the active site.

3.4. Interactions at the entrance to the gorge

At the top of the gorge, the indanone ring stacks against the 6-membered ring of the indole moiety of Trp279, in the peripheral binding site, in a classic parallel π - π interaction. The fact that the binding of E2020 is strongly dependent on interaction with Trp279 and Phe330, which are absent in *BChE*, may explain its high relative specificity for *AChE* versus *BChE*. The carbonyl function on the indanone is not in direct contact with the protein, but appears to make a water-bridged contact with F288N.

3.5. Only one enantiomer of E2020 is bound to *AChE*

The reported pharmacological studies on E2020 emphasize that both enantiomers are active, and exhibit similar, but not identical, binding affinities for *AChE* [11, 19]. Since we used the racemate in our crystallographic study, we expected either to find evidence, in the form of electron density, for the presence of both enantiomers in the crystal structure, or partial disorder which would support the presence of both. Yet, when we attempted to fit a number of plausible inhibitor conformations within the active-site gorge, a unique fit to the experimental electron density was found for one conformation of the R form, which is very similar to our energetically minimized E2020 conformation, with the indanone carbonyl group pointing towards F288N as mentioned above. Thus, *TcAChE* appears to have bound the R form selectively, despite the similarity in binding constants. Selective binding cannot be due to a limitation in the amount of the S form, since the racemate was soaked into the crystal at 10 mM, i.e.,

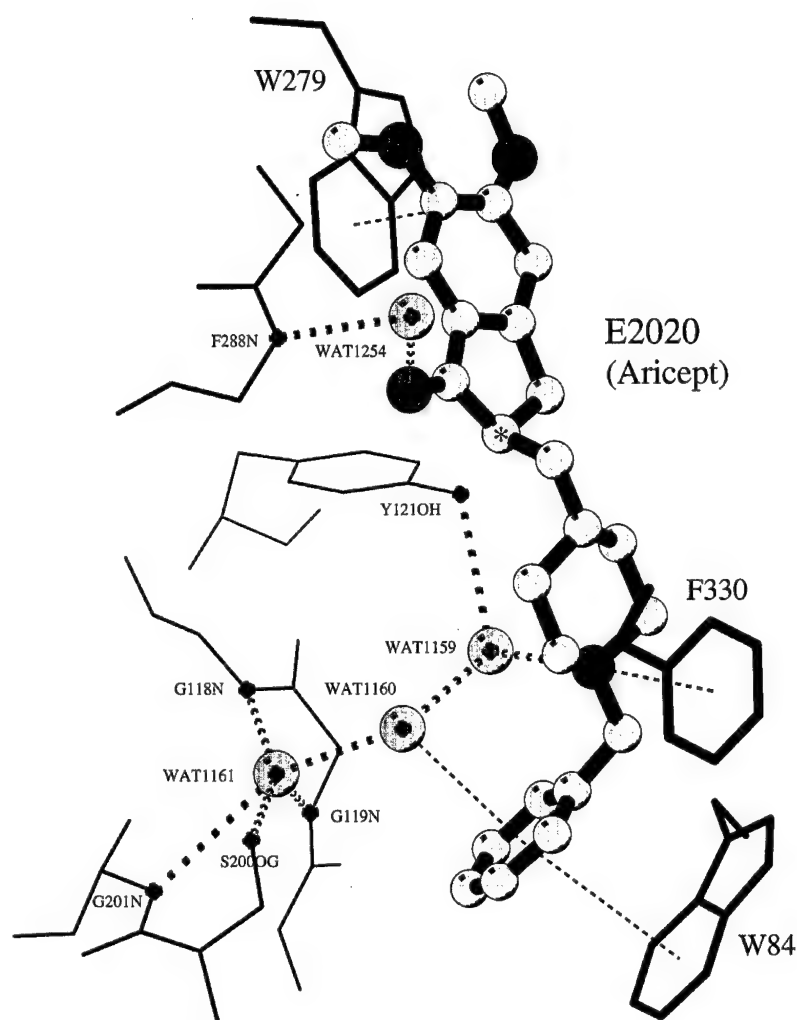


Figure 1. Binding modes of E2020 to TcAChE. E2020 are displayed as a ball-and-stick model (chiral center marked with black star); direct binding residues are represented as dark grey sticks; water-mediated binding residues as light grey sticks; water molecules as light grey balls; 'standard' H-bonds as heavy dashed lines; aromatic H-bonds, -cation and -stacking as light dashed lines.

at a great molar excess. Although the two of E2020 interconvert readily in aqueous solution, via a ketoenol intermediate [14], it is not immediately obvious what could cause such a preferential binding of one form, taking into account their similar affinities. It should, however, be borne in mind that not only are the ligand and active site chiral, but also the entire lattice of the crystalline enzyme. Such a chiral environment might cause the stereoisomers to tautomerize at different rates. Based on steric considerations, it appears that both the R and S enantiomers could bind in very similar conformations, with only the position of the carbonyl function on the 5-membered ring of the indanone moiety distinguishing between them. A model which we built displaying a carbonyl on the other side of the indanone,

representing the S form, does not make contact with any protein atom, and thus might be bound less tightly than the observed R form, even though this is inconsistent with the published inhibition data [11]. Furthermore, the 4.0 Å link between the indanone carbonyl and F288N, whether bridged by a water molecule or not, combined with the fact that the carbonyl 'nests' amongst three aromatic systems of residues Phe288, Phe290 and Phe331, might be sufficient to introduce a bias in favor of preferential binding of the R form. As already mentioned, R-S interconversion, via tautomerization, is known to occur, and might indeed take place under the experimental conditions employed. Thus, one explanation for the inhibitory potency of the S form would invoke AChE-induced S-to-R tautomerization.

3.6. Possible effect on amyloid deposition

Recently, evidence was presented that AChE may contribute to the generation of amyloid proteins and/or physically affect the process of fibril assembly which results in the formation of the senile plaques characteristic of AD [10]. The acceleration in fibril assembly produced by AChE could be retarded by the peripheral site inhibitor, propidium, and it was suggested that a hydrophobic environment close to the peripheral binding site might be involved in this process [17]. This raises the possibility that E2020, which our data clearly show as stacking against Trp279, might also moderate the rate of fibril formation. Many of the compounds synthesized and tested by the Eisai company involved modification of this segment of the molecule [3, 4]. It should be noted, however, that the screening which they performed involved assessment of affinity for AChE, together with selectivity for AChE relative to BChE, not a possible effect on amyloid fibril assembly or deposition.

Acknowledgments

This research was supported by the European Union, by the U.S. Army Medical Research and Materiel Command, under Contract No. DAMD17-97-2-7022, and by the Kimmelman Center for Biomolecular Structure and Assembly. The generous support of Tania Friedman is gratefully acknowledged. I.S. is Bernstein-Mason Professor of Neurochemistry.

References

- [1] Axelsen P.H., Harel M., Silman I., Sussman J.L., Structure and dynamics of the active site gorge of acetylcholinesterase: synergistic use of molecular dynamics simulation and X-ray crystallography, *Prot. Sci.* 3 (1994) 188–197.
- [2] Brünger A.T., Kuriyan J., Karplus M., Crystallographic R factor refinement by molecular dynamics, *Science* 235 (1987) 458–460.
- [3] Cardozo M.G., Iimura Y., Sugimoto H., Yamanishi Y., Hopfinger A.J., QSAR analyses of the substituted indanone and benzylpiperidine rings of a series of indanone benzylpiperidine inhibitors of acetylcholinesterase, *J. Med. Chem.* 35 (1992) 584–589.
- [4] Cardozo M.G., Kawai T., Imura Y., Sugimoto H., Yamanishi Y., Hopfinger A.J., Conformational analyses and molecular shape comparisons of a series of indanone benzylpiperidine inhibitors of acetylcholinesterase, *J. Med. Chem.* 35 (1992) 590–601.
- [5] Dougherty D., Cation- π interaction in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp, *Science* 271 (1996) 163–1684.
- [6] Gauthier S., Gauthier L., in: Giacobini E., Becker R. (Eds.), *Cholinergic Basis for Alzheimer Therapy*, Birkhäuser, Boston, 1991, pp. 224–230.
- [7] Giacobini E., Becker R., *Alzheimer Disease: Therapeutic Strategies* Birkhäuser, Boston, 1994.
- [8] Harel M., Schalk I., Ehret-Sabatier L., Bouet F., Goeldner M., Hirth C., Axelsen P., Silman I., Sussman J.L., The X-ray structure of a transition state analog complex reveals the molecular origins of the catalytic power and substrate specificity of acetylcholinesterase, *Proc. Natl. Acad. Sci. USA* 90 (1993) 9031–9035.
- [9] Harel M., Quinn D.M., Nair H.K., Silman I., Sussman J.L., Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase, *J. Am. Chem. Soc.* 118 (1996) 2340–2346.
- [10] Inestrosa N.C., Alvarez A., Perez C.A., Moreno R.D., Vicente M., Linker C., Casanueva O.I., Soto C., Garrido J., Acetylcholinesterase accelerates assembly of amyloid-beta-peptides into Alzheimer's fibrils: possible role of the peripheral site of the enzyme, *Neuron* 16 (1996) 881–91.
- [11] Inoue A., Kawai T., Wakita M., Iimura Y., Sugimoto H., Kawakami Y., The simulated binding of (+/-)-2,3-dihydro-5,6-dimethoxy-2-((L-phenylmethyl)-4-piperidinyl)methyl)-1H-inden-1-one hydrochloride (E2020) and related inhibitors to free and acetylated acetylcholinesterase and corresponding structure-activity analyses, *J. Med. Chem.* 39 (1996) 4460–4470.
- [12] Laskowski R.A., SURFNET: a program for visualizing molecular surfaces, cavities and intermolecular interactions, *J. Mol. Graph.* 13 (1995) 323–330.
- [13] Main A.R., in: Goldberg A.M., Hanin I. (Eds.), *Biology of Cholinergic Function*, Raven, New York, 1976, pp. 269–353.
- [14] Matsui K., Oda Y., Ohe H., Tanaka S., Asakawa N., Direct determination of 2020 enantiomers in plasma by liquid chromatography-mass spectrometry and column-switching techniques, *J. Chromatogr. A* 694 (1995) 209–218.
- [15] Quinn D.M., *Chem. Rev.* 87 (1987) 955–975.
- [16] Raves M.L., Harel M., Pang Y.-P., Silman I., Kozikowski A.P., Sussman J.L., Structure of acetylcholinesterase complexed with the nootropic alkaloid, (-)-huperzine A, *Nature Struct. Biol.* 4 (1996) 57–63.
- [17] Reyes A.E., Perez D.R., Alvarez A., Garrido J., Gentry M.K., Doctor B.P., Inestrosa N.C., A monoclonal antibody against acetylcholinesterase inhibits the formation of amyloid fibrils induced by the enzyme, *Biochem. Biophys. Res. Commun.* 232 (1997) 652–655.
- [18] Sugimoto H., Tsuchiya Y., Sugumi H., Higurashi K., Karibe N., Imura Y., Sasaki A., Araki S., Yamanishi Y., Yamatsu K., Synthesis and antiacetylcholinesterase activity of 1-benzyl-4-((5,6-dimethoxy-1-indanon-2-yl)methyl)piperidine hydrochloride (E2020) and related compounds, *J. Med. Chem.* 35 (1992) 4542–4548.
- [19] Sugimoto H., Iimura Y., Yamanishi Y., Yamatsu K., Synthesis and structure-activity-relationships of acetylcholinesterase inhibitors - 1-benzyl-4-(2-phthalimidoethyl)piperidine and related derivatives, *Bioorg. Med. Chem. Lett.* 2 (1992) 871–876.
- [20] Sussman J.L., Harel M., Frolow F., Oefner C., Goldman A., Tokar L., Silman I., Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein, *Science* 253 (1991) 872–879.
- [21] Taylor P., in: Gilman A.G., Nies A.S., Rall T.W., Taylor P. (Eds.), *The Pharmacological Basis of Therapeutics*, Macmillan, New York, 5th edn., 1990, pp. 131–150.
- [22] Taylor P., Lappi S., Interaction of fluorescence probes with acetylcholinesterase. The site and specificity of propidium binding, *Biochemistry* 14 (1975) 1989–1997.
- [23] Thomsen T., Kewitz H., Selective inhibition of human acetylcholinesterase by galanthamine in vitro and in vivo, *Life Sci.* 46 (1990) 1553–1558.

Targeting acetylcholinesterase molecules to the neuromuscular synapse

Richard L. Rotundo^a, Sasana G. Rossi^a, H. Benjamin Peng^b

^a*Departments of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, Florida 33136, USA*

^b*University of North Carolina, Chapel Hill, North Carolina 27599, USA*

Abstract — The functional integrity of the neuromuscular synapse requires that sufficient numbers of acetylcholinesterase (AChE) molecules be localized on the specialized extracellular matrix between the nerve terminal and the post-synaptic membrane. Multiple interrelated levels of regulation are necessary to accomplish this complex task including the spatial and temporal restriction of AChE mRNA expression within the muscle fiber, local translation and assembly of AChE polypeptides, and focused accumulation of AChE molecules on the extracellular matrix. This is accomplished in part through the organization of other extracellular matrix molecules into a complex which further associates with acetylcholine receptors and their accompanying molecules. Finally, the mature neuromuscular junction contains molecules which can act as receptors for the attachment of AChE which in turn may allow for the turnover of this enzyme at the synapse. This brief review will focus mainly on contributions from our laboratory towards understanding the mechanisms involved in organizing AChE molecules at the neuromuscular synapse. (©Elsevier, Paris)

Résumé — L'adressage des molécules d'acétylcholinestérase à la jonction musculaire. L'intégrité fonctionnelle de la synapse neuromusculaire nécessite que suffisamment de molécules d'acétylcholinestérase (AChE) soient localisées sur la matrice extracellulaire spécialisée entre la terminaison nerveuse et la membrane post-synaptique. Plusieurs niveaux de régulation sont nécessaires pour réaliser cette tâche et incluent la restriction spatiale et temporelle de l'expression de l'ARNm codant l'AChE dans la fibre musculaire, la traduction et l'assemblage des polypeptides d'AChE et la focalisation des molécules d'AChE dans la matrice extracellulaire. Ceci est réalisé en partie par l'organisation avec d'autres molécules de la matrice extracellulaire dans un complexe qui est ensuite associé aux récepteurs d'acétylcholine. Finalement, la jonction neuromusculaire mature comporte d'autres molécules qui peuvent agir comme un récepteur pour l'ancrage de l'AChE et pour le renouvellement de l'enzyme à la synapse. Ce bref compte-rendu est focalisé principalement sur les contributions de notre laboratoire à la compréhension des mécanismes engagés dans l'organisation des molécules d'acétylcholinestérase à la jonction neuromusculaire. (©Elsevier, Paris)

extracellular matrix / perlecan / basal lamina / localized gene expression

1. Introduction

The physiological function of acetylcholinesterase (AChE) at the neuromuscular junction (NMJ) is to terminate neurotransmission. To accomplish this, AChE molecules must be properly localized at the synapse where they are organized on the specialized extracellular matrix interposed between the nerve terminal and the post-synaptic membrane. The expression of AChE in nerves and muscle, the appearance of this enzyme at synapses or other specialized locations on the cell surface, is thus the endpoint in a series of events, each of which can be viewed as potentially or actually regulatory. However, it should be remembered that unless these molecules are correctly localized within their appropriate domains on the cell surface, their physiological function is abrogated. Targeting AChE to the neuromuscular synapse therefore includes all the events, from the initial transcription of the AChE gene to the final attachment of the assembled AChE molecules on the synaptic basal lamina. Here we shall review the major steps in targeting AChE to the synaptic basal lamina and our understanding of the mo-

lecular events that ensure that AChE molecules are correctly organized at nerve-muscle synapses.

2. Differentiation of muscle and the transcriptional and post-transcriptional control of AChE

Skeletal muscle fibers are unique in many respects because of their large size and complex organization. For example, skeletal muscle fibers can be upwards of several millimeters long, and normally contain hundreds or thousands of nuclei. The neuromuscular synapse, on the other hand, covers but a small portion of the fiber surface, usually less than 0.1%, and therefore molecules destined for the NMJ must arise from a correspondingly smaller region of the cell.

Multinucleated skeletal muscle fibers arise from the fusion of many mononucleated myoblasts which themselves do not express AChE [4]. Upon fusion to form multinucleated myotubes the cells increase their expression of AChE mRNA by processes which can involve either stabilization of the transcripts [4] or increases in the rate of transcription ([19]; in pre-

paration) depending upon the species or cell type used. At this point the AChE protein is expressed as active enzyme. AChE transcript levels are initially high throughout the length of the myotube. With further differentiation of the myotubes to form mature contracting skeletal muscle fibers, the levels of AChE transcripts decrease once again to very low levels along the length of the fiber with the exception of the innervated region where elevated levels persist throughout the lifespan of the organism [6, 8, 11]. Thus activity of the muscle fibers can suppress expression of the AChE transcripts by at least two different mechanisms, transcriptional controls and transcript destabilization. However, the mechanisms responsible for maintaining elevated levels of AChE mRNA expression, as well as other synapse-specific proteins, at sites of nerve-muscle contact are as yet unknown.

3. Compartmentalization of AChE expression in muscle

Compartmentalization in skeletal muscle refers to the fact that many cellular and biochemical functions within these complex multinucleated cells appear to be carried out in a spatially-restricted manner, as if each nucleus were still a mononucleated cell acting within the context of an organized tissue. That is to say that, although many nuclei are receiving the same signals at any one time and thus behaving in a coordinated manner, each nucleus is capable of acting somewhat independently. Likewise, gene expression in multinucleated skeletal muscle fibers also appears to be somewhat restricted in that transcripts from an individual nucleus tend to remain in the vicinity of that nucleus and be translated locally on either the rough endoplasmic reticulum associated with that nucleus or cytoplasmically [12, 14, 20] (for review see [5]).

Evidence that the transcription and translation of AChE is compartmentalized comes from experiments in which mosaic myotubes were prepared from two populations of myoblasts, each expressing a different allelic variant of the AChE catalytic subunit [20]. These allelic variants, α and β , differ by approximately 10 kDa and can easily be distinguished by their mobilities on SDS polyacrylamide gels. Moreover, these subunits assemble to form disulfide-bonded dimers which can also be resolved by SDS gel electrophoresis to quantitate the relative proportions of the $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$ dimers. Myotubes resulting from the fusion of all heterozygous myoblasts express the several dimeric AChE forms in the ratio of 25:50:25, as would be predicted assuming random assembly in the rough endoplasmic reticulum. In contrast, dimeric AChE forms assem-

bled in multinucleated myotubes arising from the fusion of equal numbers of $\alpha\alpha$ and $\beta\beta$ myoblasts resulted in more than 90% of the disulfide-bonded dimers being in the $\alpha\alpha$ and $\beta\beta$ forms. Labeling of the nuclei with tritiated thymidine, followed by autoradiography, showed that the nuclei were randomly mixed within the myotubes and therefore the results were not due to segregation of the nuclei expressing each allelic variant. These experiments show that once expressed, AChE transcripts are highly localized and tend not to diffuse very far from the nucleus of origin. Moreover, the transcripts are locally translated, and the subunits locally assembled in the rough endoplasmic reticulum prior to export to the cell surface. These observations are important for understanding the localization of AChE mRNA to the region of the neuromuscular junction.

Much more is known about the biogenesis and localization of AChE in tissue-cultured myotubes because of the ease with which the culture system can be experimentally manipulated. Once the AChE catalytic subunits are assembled into complex oligomeric forms (see Massoulié et al., this volume) they are transported and either secreted or localized on the surface of the myotubes [21]. At least some of the cell surface AChE is concentrated in clusters on the cell surface, a subset of which also contain ACh receptors. These clusters tend to localize within one nuclear diameter (about 20 μm) of a nucleus and thus form part of what can be called a nuclear domain [15]. Studies using mosaic mouse-quail myotubes showed that the AChE expressed by a specific nucleus is clustered on the region of membrane/extracellular matrix overlying that nucleus. Thus each nucleus is responsible for assembling the ECM on its overlying plasma membrane and targeting the AChE molecules to that domain.

4. Organization of cell surface AChE molecules in skeletal muscle

Cell surface AChE molecules can be classified into several types depending upon their oligomeric organization, post-translational modifications, and association with non-catalytic subunits (for review see [10]). The specific forms expressed depend in part upon the specific transcripts expressed and the cell type that expresses them. In tissue cultured skeletal muscle, most, if not all of the AChE forms that reach the cell surface appear to be either collagen-tailed forms that are rapidly attached to the surrounding extracellular matrix, or dimeric/ tetrameric forms that are secreted into the surrounding milieu. In vivo, tetramers appear to be secreted under some physiological conditions. The collagen-tailed AChE form that associates with the synaptic basal lamina

has been more difficult to study once it is externalized by the cell because of its tight association with the cell surface [16]. For this reason it is still not clear exactly how it becomes attached to the ECM, and even less is known about the mechanisms that specifically organize the AChE molecules on the extracellular matrix.

In the absence of nerves, spontaneous clusters of AChE molecules will form on the surface of tissue-cultured myotubes [15, 23]. These clusters consist predominantly, if not uniquely, of the collagen-tailed A₁₂ AChE form [15]. It appears that following externalization the collagen-tailed AChE can undergo transient electrostatic interactions with the glycan portion of a heparan sulfate proteoglycan before immobilization on the extracellular matrix [2, 18]. Although normally the collagen-tailed AChE is not found secreted into the medium, inclusion of heparin in the culture medium results in secretion of this complex enzyme form. The interpretation of these experiments is that the soluble heparin, interacting with the heparin-binding domains of the collagen tail, prevents the transient interactions between the tail and the extracellular matrix from occurring.

Recently, progress has been made in identifying the molecule(s) to which AChE might attach on the synaptic basal lamina ([17]; Peng et al., submitted). AChE co-localizes on the surface of tissue-cultured myotubes as well as the adult neuromuscular junction with a specific heparan sulfate proteoglycan. This HSP has now been identified as perlecan [13] and is the same HSP recognized by mAb 33 described by Bayne et al. [1] (Peng et al., submitted). The collagen-tailed A₁₂ AChE form can bind directly to perlecan, whereas the G₄/G₂ globular forms cannot. Although these studies do not prove that perlecan is the sole binding site for AChE, in fact other NMJ-localized proteoglycans such as agrin may also act as an AChE acceptor, they do suggest that it may be a major binding site for localizing this enzyme to sites of nerve-muscle contact.

Together, these studies suggest a mechanism for clustering AChE molecules at developing neuromuscular junctions. The collagen-tailed AChE molecules can bind to the sulfated glycan side chains of perlecan, which in turn binds to α -dystroglycan [13]. The α -dystroglycan itself is part of a large transmembrane complex which includes dystrophin on the cytoplasmic side of the membrane and can interact with several components of the extracellular matrix on the extracellular side. This complex is mobile in the plane of the lipid bilayer and can thus translocate together with other associated macromolecules. Studies in which endogenous cell surface AChE has been labeled with fluorescent fasciculin, or exogenous collagen tailed AChE added followed

by detection with appropriate antibodies, show that spinal chord neurons or growth factor-coated beads can induce the accumulation of AChE at newly formed synapses in culture. This induced clustering of AChE could provide not only a mechanism for localizing this enzyme to domains of ACh receptor accumulation, it also provides a mechanism for initiating formation of the specialized extracellular matrix that forms the synaptic basal lamina.

5. Targeting AChE to the mature neuromuscular junction

AChE has been known to be concentrated at the neuromuscular junction for many decades, since the early studies by Marnay and Nachmansohn [9]. That proper innervation is also required to maintain normal levels of this enzyme at the synapse has also been known for a long time [3]. However, remarkably, very little is known about the dynamics of this enzyme at the adult neuromuscular synapse, or at any other cholinergic synapse for that matter. Although the density and distribution of AChE has been carefully measured at the NMJ, only one study has accurately determined its half-life in vivo using metabolic labeling techniques [7]. In fact, it was not until fairly recently that the almost irreversible attachment of the enzyme to the synaptic basal lamina was appreciated [16, 18].

Once the neuromuscular junction with its specialized extracellular matrix has formed, it is unlikely that many molecules will be capable of freely diffusing in the plasma membrane. Likewise for the extracellular matrix itself, where constituent molecules are often covalently cross-linked and the entire assembly is remarkably resistant to extraction even with chaotropic agents such as 8 M urea or 5 M guanidine HCl and strong ionic detergents. The mechanisms that allow for the orderly removal and replacement of these extracellular matrix molecules must therefore be complex.

For the collagen-tailed A₁₂ AChE, it would appear that the assembled molecules must be secreted intact and somehow diffuse to their sites of attachment on the synaptic basal lamina. This is based on the observations that collagen-tailed AChE purified from avian muscle can be 'transplanted' onto the frog neuromuscular junction by simply incubating sections of frog muscle, or even isolated basal lamina, with the enzyme [22]. Unlike higher vertebrates, the frog does not have an accumulation of 'junctional' nuclei beneath the neuromuscular junction which can extend out along the muscle fiber for over 300 μ m. Thus in some cases AChE molecules must diffuse over distances greater than 100 μ m from their sites of secretion to sites of attachment on the synaptic

basal lamina. In higher vertebrates, where there is an accumulation of nuclei beneath the neuromuscular junction, it would appear that secretion followed by some diffusion would still be necessary for the AChE forms to reach their attachment sites on the basal lamina.

6. Conclusions

Ensuring that the appropriate concentration of AChE is maintained at the neuromuscular synapse requires multiple coordinated steps including spatial regulation of gene expression in the multinucleated skeletal muscle fibers. During development the expression of AChE transcripts becomes suppressed in non-innervated regions of the fibers and maintained at high levels in the vicinity of the neuromuscular junctions. This ensures that the translated proteins are assembled in the region of the neuromuscular synapse, and that the newly-synthesized and assembled AChE molecules are transported to the plasma membrane and released into the junctional space. Probably only the A₁₂ forms are retained and attached to the synaptic basal lamina where they associate with heparan sulfate proteoglycans such as perlecan and possibly agrin. During initial stages of synapse formation the AChE molecules bound to HSPs may be mobile in the plasma membrane and be capable of diffusing into clusters containing other extracellular matrix molecules as well as acetylcholine receptors and associated molecules. This accumulation of extracellular, transmembrane, and cytoplasmic synaptic components may be the earliest stages of formation of the complex specialized synaptic basal lamina. Subsequently, at the mature NMJ, A₁₂ AChE molecules may be released where they bind to pre-formed sites established by the HSPs, a form of molecular parking lot where the enzyme can attach and turn over as necessary.

References

- [1] Bayne E.K., Anderson M.J., Fambrough D.M., Extracellular matrix organization in developing muscle: correlation with acetylcholine receptor aggregates, *J. Cell. Biol.* 99 (1984) 1486–1501.
- [2] Brandan E., Maldonado M., Garrido J., Inestrosa N.C., Anchorage of collagen-tailed acetylcholinesterase to the extracellular matrix is mediated by heparan sulfate proteoglycans, *J. Cell Biol.* 101 (1985) 985–992.
- [3] Couteaux R., Nachmansohn D., Cholinesterase at the endplates of voluntary muscle after nerve degeneration, *Nature* 142 (1938) 481.
- [4] Fuentes M.E., Taylor P., Control of acetylcholinesterase gene-expression during myogenesis, *Neuron* 10 (1993) 679–687.
- [5] Hall Z., Ralston E., Nuclear domains in muscle cells, *Cell* 59 (1989) 771–772.
- [6] Jasmin B.J., Lee R.K., Rotundo R.L., Compartmentalization of acetylcholinesterase mRNA and enzyme at the vertebrate neuromuscular junction, *Neuron* 11 (1993) 467–477.
- [7] Kasprzak H., Salpeter M.M., Recovery of acetylcholinesterase at intact neuromuscular junctions after in vivo inactivation with di-isopropylfluorophosphate, *J. Neurosci.* 5 (1985) 951–955.
- [8] Legay C., Huchet M., Massoulié J., Changeux J.-P., Developmental regulation of acetylcholinesterase transcripts in the mouse diaphragm: alternative splicing and focalization, *Eur. J. Neurosci.* 7 (1995) 1803–1809.
- [9] Marnay A., Nachmansohn D., Cholinesterase in voluntary muscle, *J. Physiol. (Lond.)* 92 (1938) 37–47.
- [10] Massoulié J., Pezzementi L., Bon S., Krejci E., Vallette F.M., Molecular and cellular biology of cholinesterases, *Progr. Neurobiol.* 41 (1993) 31–91.
- [11] Michel R.N., Vu C.Q., Tetzlaff W., Jasmin B.J., Neural regulation of acetylcholinesterase mRNAs at mammalian neuromuscular synapses, *J. Cell Biol.* 127 (1995) 1061–1069.
- [12] Pavlath G.K., Rich K., Webster S.G., Blau H.M., Localization of muscle gene products in nuclear domains, *Nature* 337 (1989) 570–573.
- [13] Peng H.B., Ali A.A., Daggett D.F., Rauvala H., Hassell J.R., Smalheiser N.R., The relationship between perlecan and dystroglycan and its implication in formation of the neuromuscular junction, *Cell Adh. Comm.* (1998), in press.
- [14] Ralston E., Hall Z.W., Restricted distribution of mRNA produced from a single nucleus in hybrid myotubes, *J. Cell Biol.* 119 (1992) 1063–1068.
- [15] Rossi S.G., Rotundo R.L., Cell surface acetylcholinesterase molecules on multinucleated myotubes are clustered over the nucleus of origin, *J. Cell Biol.* 119 (1992) 1657–1667.
- [16] Rossi S.G., Rotundo R.L., Localization of 'non-extractable' acetylcholinesterase to the vertebrate neuromuscular junction, *J. Biol. Chem.* 268 (1993) 19152–19159.
- [17] Rossi S.G., Rotundo R.L., Transient interactions between acetylcholinesterase and localized proteoglycans prior to immobilization on the extracellular matrix, Abstract, American Society for Cell Biology Meeting, Washington, D.C., 1995.
- [18] Rossi S.G., Rotundo R.L., Transient interactions between collagen-tailed acetylcholinesterase and sulfated proteoglycans prior to immobilization on the extracellular matrix, *J. Biol. Chem.* 271 (1996) 1979–1987.
- [19] Rossi S.G., Rotundo R.L., Regulation of acetylcholinesterase mRNA during differentiation of quail skeletal muscle in culture, abstract, American Society for Neuroscience, 1997.
- [20] Rotundo R.L., Nucleus-specific translation and assembly of acetylcholinesterase in multinucleated muscle cells, *J. Cell Biol.* 110 (1990) 715–719.
- [21] Rotundo R.L., Fambrough D.M., Synthesis, transport, and fate of acetylcholinesterase in cultured chick embryo muscle cells, *Cell* 22 (1980) 583–594.
- [22] Rotundo R.L., Rossi S.G., Anglister L., Transplantation of quail acetylcholinesterase to the frog neuromuscular junction, *J. Cell Biol.* 136 (1997) 367–374.
- [23] Wallace B.G., Nitkin R.M., Reist N.E., Fallon J.R., Moayeri N.N., McMahan U.J., Aggregates of acetylcholinesterase induced by acetylcholine receptor-aggregating factor, *Nature* 315 (1985) 574–577.

Imprinting of hippocampal metabolism of choline by its availability during gestation: Implications for cholinergic neurotransmission

Jan Krzysztof Blusztajn, Jennifer Marie Cermak, Thomas Holler, Darrell A. Jackson

*Department of Pathology and Laboratory Medicine, Boston University School of Medicine,
85 East Newton Street, Room M1009, Boston, MA 02118, USA*

Abstract — Choline supplementation during the second half of the gestational period in rats permanently improves visuospatial memory. Choline availability during this period also alters the turnover of choline and acetylcholine in the hippocampus in 3–4-week-old animals in a complex pattern consistent with the notion that cholinergic neurotransmission is enhanced by prenatal choline supplementation. (©Elsevier, Paris)

Résumé — Effet à long terme du métabolisme de la choline dans l'hippocampe par sa disponibilité pendant la gestation : implications dans la neurotransmission cholinergique. L'apport de choline pendant la seconde moitié de la gestation chez le rat améliore de façon permanente la mémoire visuospatiale. La disponibilité en choline pendant cette période altère également le turn-over de la choline et de l'acétylcholine dans l'hippocampe chez les animaux de 3–4 semaines. Ceci est en accord avec l'idée que la neurotransmission cholinergique est augmentée par l'apport prénatal de choline. (©Elsevier, Paris)

acetylcholine / phosphatidyl choline / memory / nutrition

Choline is an essential nutrient for animals and humans [27] and the 1998 report on B vitamins of the Food and Nutrition Board of the Institute of Medicine of the US National Academy of Sciences, for the first time, issued recommendations for the Adequate Intake of choline [10]. Recent studies have shown that the availability of choline during a specific prenatal period in rats (embryonic (E) days 11 through 17 of the 22-day gestation period) has profound effects on cognitive performance throughout the lifespan. In brief, prenatally choline-supplemented adult rats are characterized by improved performance relative to prenatally-deficient and control animals in tasks measuring spatial memory, temporal processing, and attention [15–18, 21, 26]. In contrast, prenatally deficient animals are impaired in attentional and certain memory tasks [16]. The behavioral effects of choline availability in utero persist beyond the age of 2 years [16], which is regarded as old in the rat. Thus, prenatal supplementation with choline prevents the characteristic memory decline of old age. These data indicated that prenatal availability of choline permanently influences brain organization and function. Data consistent with this hypothesis have been obtained using neuroanatomical [25], neurophysiological [20], and neurochemical [6, 12] approaches. For example, studies of hippocampal long-term potentiation (LTP), a measure of synaptic plasticity and a model for learning and memory [2, 8], showed that in young adult rats prenatally supplemented with choline, induction of LTP was facilitated due to a reduction in the stimulus

threshold for LTP generation, while the stimulus threshold for LTP generation was increased in prenatally choline-deficient animals [20].

The neurochemical mechanisms by which choline supplementation in utero leads to an improvement in memory are not known. Choline serves several biological functions [27]: 1) it is the precursor of the neurotransmitter, acetylcholine (ACh), in cholinergic neurons [3]; 2) it is the precursor of phosphatidylcholine (PC) and sphingomyelin, which are structural phospholipids in biological membranes, and which act as precursors for intracellular messengers such as diacylglycerol or ceramide; 3) it is the precursor of two signaling lipids, sphingosylphosphocholine and platelet-activating factor (PAF). The latter appears to be important in brain development because mutations in PAF acetylhydrolase (the enzyme that inactivates PAF) cause Miller-Dieker lissencephaly [11], a genetic disorder characterized by smooth cerebral hemispheres; and 4) it can be enzymatically oxidized to betaine and the methyl groups of betaine can then be used to resynthesize methionine from homocysteine, thereby providing methionine for protein synthesis and transmethylation reactions. The latter pathway also serves as an alternative to one which uses methyltetrahydrofolate, and thus spares methyltetrahydrofolate for its role in the synthesis of nucleic acids. It is possible that choline availability in utero affects one or more of these functions during brain development, resulting in changes in the brain's organization. Since the period of E11–17 coincides with the peak of cell divi-

sion and apoptosis in the developing brain, this explanation is likely. Indeed, choline supplementation during E11-17 stimulates cell division in the embryonic brain (assessed immunohistochemically on E18 following the injection of pregnant dams on E16 with the DNA precursor bromodeoxyuridine) [22], while choline deficiency during this period increases the rate of apoptosis (observed on E18) in hippocampus and septum [13, 22], two brain regions involved in memory processing.

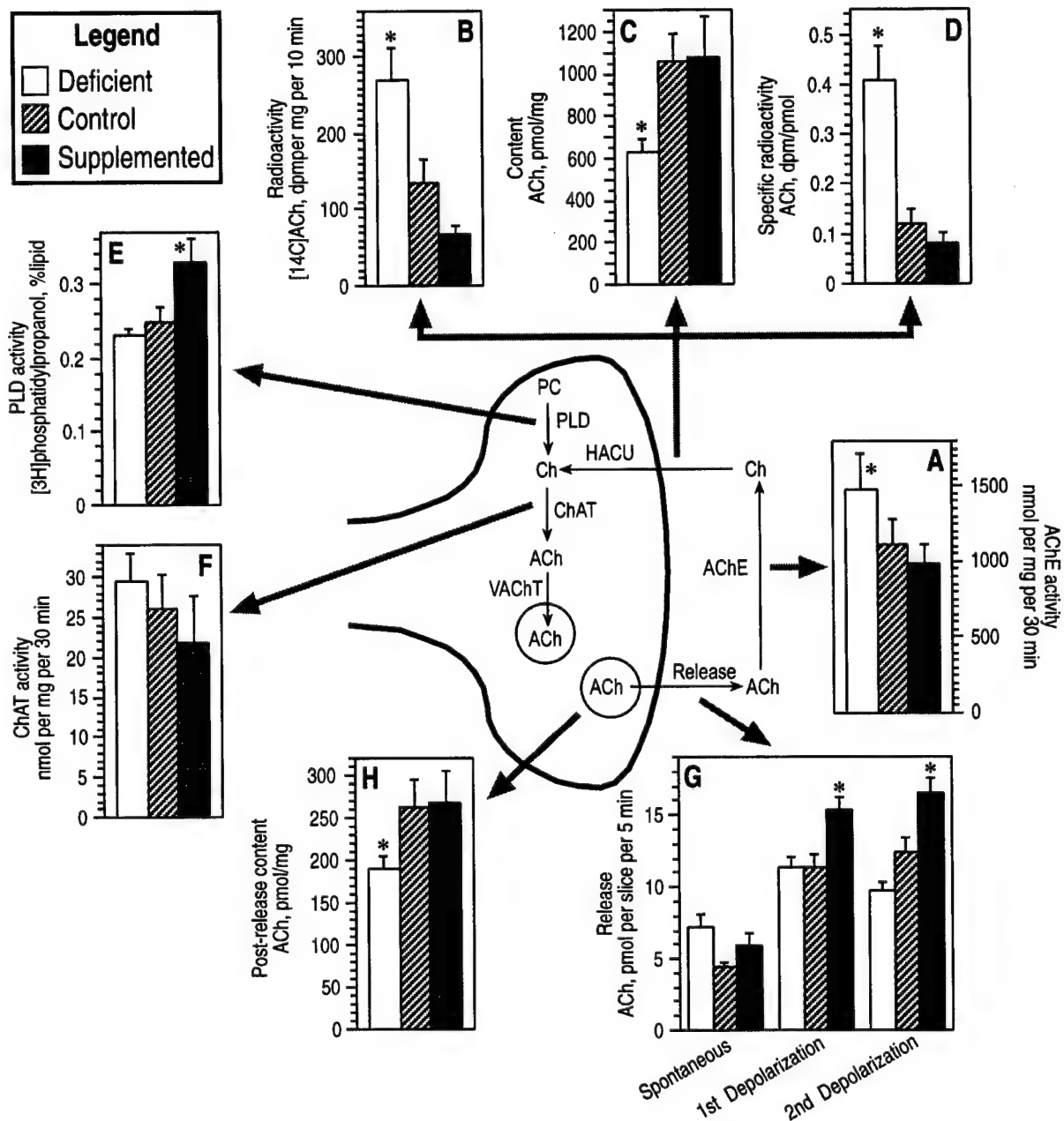
Another possible explanation for the long-term actions of prenatal choline availability on brain function that will be explored here, is that in addition to the permanent organizational changes in brain, prenatal choline status causes long-term metabolic adaptations in choline metabolism, resulting in changes in cholinergic neurotransmission. Long-term metabolic adaptations of fat and carbohydrate metabolism to the availability of carbohydrate and fat during the pre-weaning period have been observed previously [19]. This type of adaptation has been termed 'metabolic imprinting'.

In order to determine the effects of maternal choline intake on choline and ACh turnover in the hippocampus of the offspring we used pregnant rats (Sprague-Dawley *Crl:CD* (SD)BR-CD) fed varying amounts of choline during the period E11-17. We measured several indices of ACh synthesis, degradation and release, as well as the activity of a PC-hydrolyzing enzyme phospholipase D (PLD) in the offspring approximately 1 month after termination of treatment. PLD was chosen because choline liberated from PC by PLD can be used for ACh synthesis [4, 14].

The results, summarized in *figure 1*, show that prenatal choline availability altered choline and ACh turnover in the hippocampus. Prenatally choline-deficient animals displayed elevations in AChE (*figure 1A*) and ChAT (*figure 1F*) activities, and increased synthesis of ACh from choline transported by high-affinity choline uptake (HACU) (*figure 1B, D*), concomitant with reductions in hippocampal ACh content (*figure 1C, G*) and a relative inability to sustain depolarization-evoked ACh release (*figure 1H*).

Figure 1. Prenatal choline availability alters the indices of choline turnover in the hippocampus. In the center of the figure is a schematic representation of a cholinergic nerve terminal. Choline is taken up from the extracellular space by a sodium-dependent high-affinity uptake system (HACU) and then acetylated to ACh by choline acetyltransferase (ChAT). The ACh is taken up into the secretory vesicles by a specific carrier, the vesicular ACh transporter (VACHT), and then released into the synapse. Extracellular ACh is hydrolyzed by acetylcholinesterase (AChE) to free acetate and choline. The hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) provides additional choline precursor for ACh synthesis. **A-H.** The individual assays were performed as described previously [6, 12] on animals (at 21 days of age (**E**) or 27 days of age (all other panels)) whose mothers consumed no choline (deficient), 1.3 mmol per kg per day of choline (control), or 4.6 mmol per kg per day of choline (supplemented) during days 11-17 of pregnancy. **A.** Hippocampal AChE activity. AChE activity was measured in homogenates by the method of Fonnum [9] as described [6]. AChE activity was highest in the prenatally choline-deficient group as compared to control and supplemented groups ($*P < 0.05$; $P < 0.001$, respectively). **B-D.** Acetylcholine synthesis in hippocampal slices from choline transported by HACU. **B.** Incorporation of [14 C]choline into ACh. Hippocampal slices were incubated for 10 min at 37 °C with 1 μ M [14 C]choline in the presence or absence of 10 μ M hemicholinium-3, a specific inhibitor of HACU. ACh was extracted, purified by HPLC and its radioactivity was determined as described [6]. The data are reported as dpm of [14 C]ACh accumulated in the absence of hemicholinium-3 minus that accumulated in its presence, as an index of ACh synthesis from choline taken up by HACU. The amount of [14 C]choline incorporated into [14 C]ACh was increased in the prenatally choline-deficient groups compared to prenatally choline-supplemented and control groups ($*P < 0.001$). **C.** Acetylcholine content in slices incubated as in **B** in the presence of hemicholinium-3 was determined by HPLC. The amount of ACh in hippocampal slices was decreased in the prenatally choline-deficient groups compared to the prenatally choline-supplemented and control groups ($*P < 0.01$). **D.** Specific radioactivity of [14 C]ACh was obtained by dividing the values in **B** by those in **C**. The specific radioactivity of [14 C]ACh was increased in the prenatally choline-deficient groups compared to the prenatally choline-supplemented and control groups ($*P < 0.001$). **E.** Phospholipase D activity in hippocampal slices [12]. The slices were preincubated with [3 H]glycerol to label phospholipids, and subsequently incubated in the presence of 1-propanol for 30 min. [3 H]Phosphatidylpropanol, the product of PLD, was purified by TLC and its radioactivity determined and expressed as a percentage of that in the total lipids. PLD activity was higher in prenatally choline-supplemented group as compared to the control and choline-deficient groups ($*P < 0.05$). **F.** Hippocampal ChAT activity. ChAT activity was measured in homogenates by the method of Fonnum as described [6]. ChAT activity was higher in choline-deficient group compared to the prenatally choline-supplemented group ($P < 0.05$). **G.** Spontaneous and depolarization-evoked ACh release was measured as described [6]. Hippocampal slices from the prenatally choline-supplemented group had an increased 1st and 2nd depolarization-evoked ACh release compared to the prenatally choline-deficient and control groups ($*P < 0.05$). **H.** Acetylcholine content of hippocampal slices. ACh was measured in hippocampal slices by HPLC as described [6], following the 2nd depolarization period described in **G**. The prenatally choline-deficient group had reduced ACh content compared to the control and choline-supplemented groups ($*P < 0.05$).





These results indicate that in the hippocampus of prenatally choline-deficient animals ACh turnover is accelerated (i.e., there is more rapid synthesis, degradation, and choline reutilization by HACU as indicated by the high specific radioactivity of newly-synthesized ACh (figure 1D)). In contrast, prenatally choline-supplemented animals showed less pronounced changes in their hippocampal cho-

linergic system, however the direction of those changes was consistent with the above model. AChE (figure 1A) and ChAT activities (figure 1F), and ACh synthesized from choline transported by HACU (figure 1B,D), were lowest in prenatally choline-supplemented rats. However, depolarization-evoked ACh release was highest in these animals (figure 1H). The latter result, together with the reduced AChE activity,

suggest that intrasynaptic ACh concentrations and dwell times are increased, possibly resulting in enhanced cholinergic neurotransmission. The observations that ACh turnover in prenatally choline-supplemented animals is relatively slow (as indicated by low specific radioactivity of ACh newly-synthesized from exogenous choline (*figure 1D*)), but that cholinergic neurotransmission is well maintained (as evidenced by robust ACh release (*figure 1H*)), suggest that the pool of choline used for the synthesis of ACh in these animals may include that stored in membrane PC [23, 24], and may be generated by the hydrolysis of PC catalyzed by PLD [4, 14]. Consistent with the latter possibility, hippocampal PLD activity was two-fold higher in prenatally choline-supplemented rats relative to control and prenatally choline deficient animals (*figure 1E*) [12]. However, it is not yet clear if this PLD is in fact involved in supplying choline for ACh synthesis in hippocampal cholinergic nerve terminals.

Together the data show that the availability of an essential nutrient, choline, in utero causes multiple changes in its own metabolism (i.e., exerts a form of metabolic imprinting) in the hippocampus later in life. In prenatally choline-deficient animals this imprinting results in efficient recycling of choline, while in prenatally choline-supplemented rats choline recycling is reduced and there appears to be greater reliance on PC-stored choline for ACh synthesis. These adaptations seem appropriate for the periods when choline availability is altered (i.e., prenatally), however they are long-lasting, i.e., they are observed at a time when all animals consume the control diet, 1 month after the termination of treatment. The molecular mechanisms governing metabolic imprinting by choline are not known. During the period of E11-17 the brain grows rapidly due to cell division and it is possible that choline can become a rate-limiting factor in this process as it does in cell culture models [1, 5, 7, 13]. It is conceivable that when this happens, the transcriptional control of enzymes involved in choline and ACh metabolism is adjusted by unknown feedback mechanisms that operate in dividing cells, setting up the expression level of those enzymes. However, as neuronal precursor cells differentiate into neurons and become postmitotic, they no longer respond to the availability of choline and the expression of the enzymes involved in choline turnover remains at the level set during development. In the cholinergic septohippocampal pathway studied here, these changes in gene expression would have to occur in the septal neurons. Indeed, changes in the size and shape of these neurons (identified by the expression of the p75 nerve growth factor receptor) have been observed in animals prenatally supplemented with choline [25].

This model of the mechanism of metabolic imprinting by choline remains hypothetical at present, however the model is testable with available molecular tools.

Since the activities of the macromolecules involved in hippocampal ACh turnover are imprinted by choline, cholinergic neurotransmission in the adult may be differentially sensitive to the availability of choline in adulthood, based on its supply in utero. The results presented in figure 1 suggest that elevated dietary choline in adult animals that were prenatally choline-deficient would result in large increases of ACh synthesis, whereas it might have a lesser effect in prenatally choline-supplemented animals. Conversely, choline deficiency in adults that were prenatally choline-supplemented might result in a large reduction of ACh synthesis concomitant with a depletion of their PC pool. Thus prenatal choline status would impart differential vulnerability to the choline supply in adulthood.

Acknowledgments

We thank Dr. Mordechai Liscovitch and Dr. Maximilian Follettie for helpful discussions. These studies were supported by an NIA grant AG09525.

References

- [1] Albright C.D., Liu R., Bethea T.C., Da Costa K.A., Salganik R.I., Zeisel S.H., Choline deficiency induces apoptosis in SV40-immortalized CWSV-1 rat hepatocytes in culture, *FASEB J.* 10 (1996) 510-516.
- [2] Bliss T.V.P., Collingridge G.L., A synaptic model of memory: Long-term potentiation in the hippocampus, *Nature* 361 (1993) 31-39.
- [3] Blusztajn J.K., Wurtman R.J., Choline and cholinergic neurons, *Science* 221 (1983) 614-620.
- [4] Blusztajn J.K., Liscovitch M., Richardson U.I., Synthesis of acetylcholine from choline derived from phosphatidylcholine in a human neuronal cell line, *Proc. Natl. Acad. Sci. USA* 84 (1987) 5474-5477.
- [5] Blusztajn J.K., Liscovitch M., Mauron C., Richardson U.I., Ulus I., Wurtman R.J., Phosphatidylcholine as a necessary component of biological membranes and as a store of choline for acetylcholine synthesis, in: Bazan N.G., Horrocks L.A., Toffano G., (Eds.), *Phospholipids in the nervous system: biochemical and molecular pathology*, Liviana Press, Padova, 1989, pp 205-215.
- [6] Cermak J.M., Holler T., Jackson D.A., Blusztajn J.K., Prenatal availability of choline modifies development of the hippocampal cholinergic system, *FASEB J.* 12 (1998) 349-357.
- [7] Eagle H., The minimum vitamin requirements of the L and HeLa cells in tissue culture, the production of specific vitamin deficiencies, and their cure, *J. Exp. Med.* 102 (1955) 595.
- [8] Eichenbaum H., Spatial learning. The LTP-memory connection, *Nature* 378 (1995) 131-132.

- [9] Fonnum F., A rapid radiochemical method for the determination of choline acetyltransferase, *J. Neurochem.* 24 (1975) 407-409.
- [10] Food and Nutrition Board, Institute of Medicine, Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B₆, folate, vitamin B₁₂, pantothenic acid, biotin, and choline, National Academy Press, Washington, D.C.
- [11] Hattori M., Adachi H., Tsujimoto M., Arai H., Inoue K., Miller-Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor, *Nature* 370 (1994) 216-218.
- [12] Holler T., Cermak J.M., Blusztajn J.K., Dietary choline supplementation in pregnant rats increases hippocampal phospholipase D activity of the offspring, *FASEB J.* 10 (1996) 1653-1659.
- [13] Holmes-McNary M.Q., Loy R., Mar M.H., Albright C.D., Zeisel S.H., Apoptosis is induced by choline deficiency in fetal brain and in PC12, *Dev. Brain Res.* 101 (1997) 9-16.
- [14] Lee H.-C., Fellenz-Maloney M.-P., Liscovitch M., Blusztajn J.K., Phospholipase D-catalyzed hydrolysis of phosphatidylcholine provides the choline precursor for acetylcholine synthesis in a human neuronal cell line, *Proc. Natl. Acad. Sci. USA* 90 (1993) 10086-10090.
- [15] Meck W.H., Smith R.A., Williams C.L., Organizational changes in cholinergic activity and enhanced visuospatial memory as a function of choline administered prenatally or postnatally or both, *Behav. Neurosci.* 103 (1989) 1234-1241.
- [16] Meck W.H., Williams C.L., Simultaneous temporal processing is sensitive to prenatal choline availability in mature and aged rats, *Neuroreport* 8 (1997) 3045-3051.
- [17] Meck W.H., Williams C.L., Perinatal choline supplementation increases the threshold for chunking in spatial memory, *Neuroreport* 8 (1997) 3053-3059.
- [18] Meck W.H., Williams C.L., Characterization of the facilitative effects of perinatal choline supplementation on timing and temporal memory, *Neuroreport* 8 (1997) 2831-2835.
- [19] Patel M.S., Vadlamudi S.P., Johanning G.L., Overview of pup in a cup model: hepatic lipogenesis in rats artificially reared on a high-carbohydrate formula, *J. Nutr.* 123 (1993) 373-377.
- [20] Pyapali G.K., Turner D.A., Williams C.L., Meck W.H., Swartzwelder H.S., Prenatal dietary choline supplementation decreases the threshold for induction of long-term potentiation in young adult rats, *J. Neurophysiol.* 79 (1998) 1790-1796.
- [21] Schenk F., Brandner C., Indirect effect of peri- and postnatal choline treatment on place-learning abilities in rat, *Psychobiology* 23 (1995) 302-313.
- [22] Tsai A.Y., Albright C.D., Mar M.-H., Brown E., Zeisel S.H., Availability of choline modulates apoptosis and neurogenesis in fetal rat brain, *FASEB J.* 12 (1998) (abstract) 1375.
- [23] Ulus I.H., Wurtman R.J., Mauron C., Blusztajn J.K., Choline increases acetylcholine release and protects against the stimulation-induced decrease in phosphatide levels within membranes of rat corpus striatum, *Brain Res.* 484 (1989) 217-227.
- [24] Wecker L., Neurochemical effects of choline supplementation, *Can. J. Physiol. Pharmacol.* 64 (1986) 329-333.
- [25] Williams C.L., Meck W.H., Heyer D., Loy R., Hypertrophy of basal forebrain neurons and enhanced visuospatial memory in perinatally choline-supplemented rats, *Brain Res.* 794 (1998) 225-238.
- [26] Zeisel S.H., Da Costa K.-A., Franklin P.D., Alexander E.A., Lamont J.T., Sheard N.F., Beiser A., Choline, an essential nutrient for humans, *FASEB J.* 5 (1991) 2093-2098.
- [27] Zeisel S.H., Blusztajn J.K., Choline and human nutrition, *Annu. Rev. Nutr.* 14 (1994) 269-296.

TrkA antagonists decrease NGF-induced ChAT activity in vitro and modulate cholinergic synaptic number in vivo

Thomas Debeir, H. Uri Saragovi, A. Claudio Cuello*

Department of Pharmacology & Therapeutics, McGill University, 3655 Drummond Street, Montreal, PQ, Canada H3G 1Y6

Abstract — Cholinergic neurons are known to respond in vivo to the administration of nerve growth factor (NGF) by a prominent and selective increase of choline acetyl transferase activity and by cholinergic synaptogenesis in the rat brain. By using a synthetic TrkA antagonist we demonstrated that endogenously produced NGF is involved in the continual re-modeling of cholinergic neuronal connections during adulthood, acting through TrkA receptors. (©Elsevier, Paris)

Résumé — Inhibition, par des antagonistes du récepteur TrkA, des propriétés trophiques du NGF in vitro et in vivo. Dans le cerveau de rat, les neurones cholinergiques répondent in vivo au NGF exogène, par une augmentation importante et sélective de l'activité de l'acétylcholine transférase et par la formation de nouvelles synapses cholinergiques. En utilisant un antagoniste synthétique du récepteur TrkA, nous avons démontré qu'au cours de l'âge adulte, le NGF endogène participe, via le récepteur TrkA, au processus permanent de plasticité synaptique des neurones cholinergiques. (©Elsevier, Paris)

NGF/ TrkA / antagonist / cortex / cholinergic neurons / VAChT / synaptophysin

1. Introduction

Several lines of evidence indicate that nerve growth factor (NGF) plays an important physiological role in regulating the phenotypic development and growth of the septohippocampal projection neurons [4]. The administration of NGF to neonate rats in vivo or to septal neurons in culture induces a several-fold increase in the activity of the acetylcholine biosynthetic enzyme, choline acetyltransferase (ChAT) [1, 8–10]. In addition, we have also provided evidence that exogenous NGF induces cholinergic synaptogenesis and hypertrophy of presynaptic boutons in the CNS of adult animals [6, 7].

The purpose of this study was to evaluate the role of endogenous NGF in the maintenance of cholinergic terminals in the cerebral cortex of adult animals by the blockade of TrkA receptors in situ. For this, we have applied a recently developed low molecular mass TrkA receptor antagonist which has been shown to counteract NGF effects in PC12 cells and to target TrkA receptors in vivo [11, 12]. The TrkA antagonist is the cyclic peptide C(92–96) which mimics the conformation of the third beta-turn (variable region) of NGF [11]. In order to validate the effectiveness of this compound in regulating NGF-sensitive cholinergic phenotype we investigated its actions in vivo on dissociated embryonic septal cells. After validating its efficacy, we applied it by infu-

sion into the cerebral cortex and analyzed its effects on the number of presynaptic elements (cholinergic and non-cholinergic).

2. Materials and methods

2.1. Materials

In these experiments, we used a cyclic conformationally constrained peptide C(92–96) (YCTDEKQCY; [11]).

2.2. In vitro

2.2.1. Septal neuronal cultures

Cell cultures were established from the septal area of 17-day-old rat embryos (Sprague-Dawley) using procedures described by Debeir et al. [3]. Cultures of septal neurons were treated 1 day after plating. The incubation was continued until 6 days in vitro (DIV) at which time ChAT activity was evaluated.

2.2.2. Measurement of ChAT activity

At 6 DIV, ChAT activity assays were performed directly in the wells using Fonnum's method [5], where ChAT activity is defined as the radioactivity obtained in the presence of eserine minus that seen in the presence of acetylcholine esterase.

2.3. In vivo

2.3.1. Drug treatment

Adult male Wistar rats (340–360 g) were anaesthetized and then implanted with cannula according to the following coordinates from Bregma [15]: anterior/posterior –1.3 mm, lateral 3, vertical 2.2 (hindlimb area). The cannula was connected to

* Correspondence and reprints

an Alzet 2004 osmotic pump (0.25 $\mu\text{L}/\text{h}$). The C(92–96) peptide was diluted to have a delivery rate of 28 μg in 24 h. Two weeks after implantation, the pumps and tubing were removed from the anaesthetized rats. Rats were killed 2 weeks after the treatment ended.

2.3.2. Perfusion and fixation

The rats were perfused as described by Wong et al. [17].

2.3.3. Synaptophysin and VACHT immunostaining

The sections were incubated with a mouse monoclonal antibody against synaptophysin (1:40, overnight, Boehringer) or with a rabbit polyclonal antibody against VACHT (1:10000, 4 $^{\circ}\text{C}$, 72 h, gift of Dr. R. Edwards, UCSF). Immunoreactivity was detected by using a PAP reaction for synaptophysin and a Vectastain ABC kit (Vector) for VACHT.

2.3.4. Quantification of the VACHT and synaptophysin stained presynaptic boutons

The immunopositive punctae (presynaptic boutons and varicosities) are referred to here as VACHT-immunoreactive (IR) and synaptophysin-IR sites. They were detected by the image analysis system using software devised for silver grain counting (Imaging Research Inc., St. Catharine, Ontario, Canada) as described by Wong et al. [17]. To analyze the effects of the peptide on the number of boutons, a two-way ANOVA test was employed.

3. Results

The TrkA antagonist C(92–96) was shown to be effective in blocking NGF-induced cholinergic differentiation on primary cultures of dissociated embryonic septal cells. In the conditions applied, basal ChAT activity in septal neurons was increased by approximately two-fold in the presence of 40 pM NGF and by 50% with 4 pM NGF. The TrkA antagonist C(92–96), per se, did not provoke any effect on either ChAT activity or on cell density (data not shown). However, in the presence of 40 pM or 4 pM of NGF, the cyclic peptide C(92–96) prevented the neurotrophin induced effects on ChAT activity in a concentration dependent manner (figure 1). Concentrations 10^6 -fold higher than those of NGF were required for the optimal blockade of neurotrophin activity by C(92–96), demonstrating that C(92–96) can also antagonize the biological effect of NGF cholinergic phenotype in vitro.

To investigate NGF actions in vivo, C(92–96) was delivered (28 $\mu\text{g}/\text{day}$) for 2 weeks into the rat cortex (hindlimb area) via a chronically implanted cannula connected to an osmotic mini-pump. Two weeks after the cessation of treatment with C(92–96), we observed a significant depletion in the number VACHT-IR sites (–23%) in the ipsilateral cortex. No significant changes in the number of synaptophysin-immunoreactivity sites were observed (table 1,

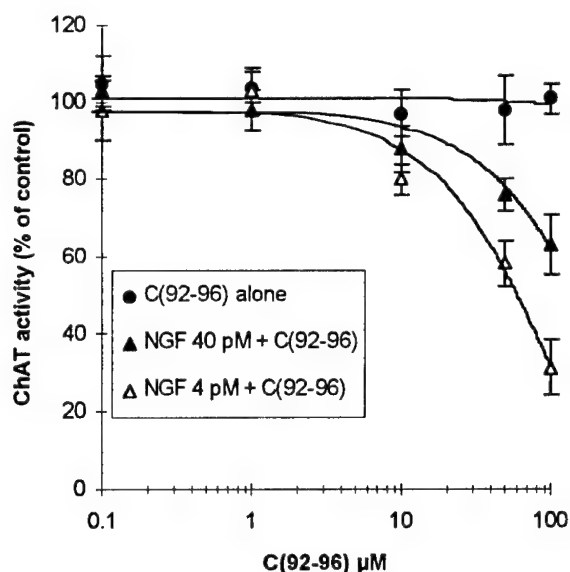


Figure 1. Effect of C(92–96) on NGF-induced cholinergic activity of septal neurons. The drugs were added to the culture medium 1 day after plating. ChAT activity was measured after 6 DIV. Values represent mean \pm S.E.M. $n = 4$.

Table 1. Effect of cortical injection of C(92–96) on the number of synaptophysin-IR and VACHT-IR sites in the hindlimb cortex.

	Contralateral		Ipsilateral	
	Synapto- physin	VACHT	Synapto- physin	VACHT
C(92-96) 28 $\mu\text{g}/\text{day}$	106%	103%	107%	77%*

The number of sites is expressed as a percent of control. For synaptophysin-IR the contralateral and ipsilateral control values are, respectively, 720 ± 25 and 744 ± 14 sites in $1000 \mu\text{m}^2$. For VACHT-IR, the control values are 26.4 ± 1.8 and 27.3 ± 1.8 sites in $1000 \mu\text{m}^2$. * $P < 0.05$ versus control.

figure 2). The treatments did not provoke any contralateral effects.

4. Discussion

Neurotrophins are responsible for inducing synaptogenesis during development (for review see [13]) and are responsible for maintaining the phenotype of some neuronal sets (for review see [14, 16]). Exogenously applied neurotrophins in the mature and fully differentiated CNS are capable of rescuing de-

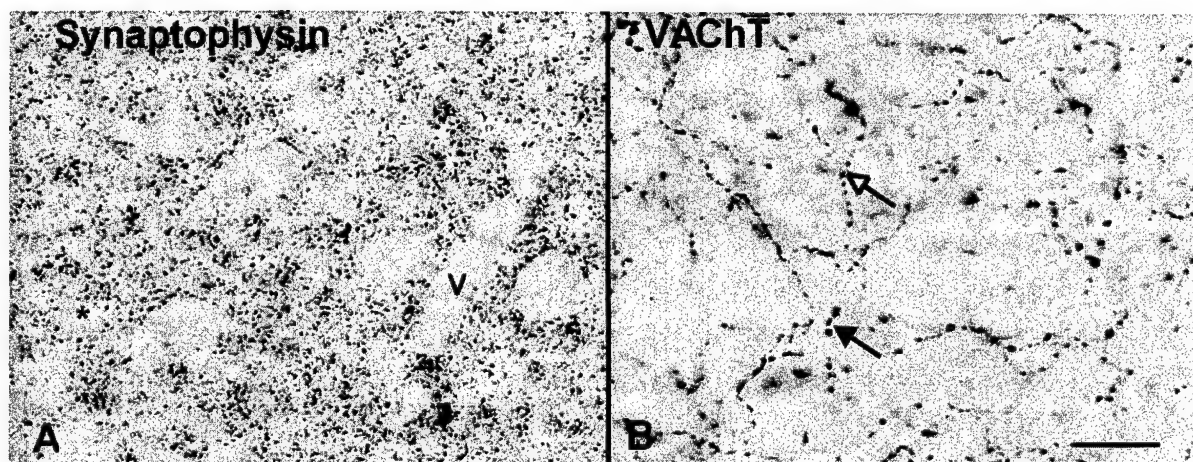


Figure 2. Synaptophysin (A) and VACHT (B) immunoreactivity in the ipsilateral cortex of control animals. Empty areas in A indicate vessels (V) or cells bodies (*) and small abundant punctae label non-cholinergic boutons. The solid arrow in B indicates three separate cholinergic boutons and the open arrow indicates an axon with multiple cholinergic varicosities. Scale bar, 20 μ m.

generating or dying neurons in the lesioned CNS (for review see [2]) and are also capable of generating new synapses in the cerebral cortex of adult, fully differentiated animals [6, 7].

The analysis of the role played by endogenous neurotrophins in the maintenance of neuronal phenotype and synaptic patterns in the adult brain has been hampered by a lack of adequate antagonists. These reagents are now becoming available. In the present study, we have shown that the blockade of the TrkA receptors of embryonic cholinergic neurons counteracts the NGF-induced differentiation and up-regulation of the acetyl choline biosynthetic enzyme. More importantly, when this TrkA blockade takes place in the cerebral cortex of otherwise naïve animals *in vivo*, it causes an effect of its own, provoking a noticeable loss of VACHT-IR but not of synaptophysin-IR sites. These results indicate that a TrkA blockade does not affect the overall, non-cholinergic, presynaptic boutons in the adult CNS. These effects appear to be rather selective in removing the NGF-sensitive, TrkA-expressing, presynaptic cholinergic boutons which account for some 3 to 5% of the total synaptic population in the cerebral cortex. These results are consistent with the expected potency of this first generation of TrkA antagonist, based on published *in vitro* data [11]. They also indicate that endogenous NGF could define the steady state number and pattern of cholinergic synapses. Furthermore, it is conceivable that neurotrophic factors in general have a role in the maintenance of synaptic contacts in the adult and fully differentiated CNS.

Acknowledgments

This work was supported by a grant from the MRC (Canada) and from Lavoisier (France). We would like to thank Dr. LeSauter for assistance with the peptides.

References

- [1] Cuello A.C., Garofalo L., Kenigsberg R.L., Maysinger D., Gangliosides potentiate *in vivo* and *in vitro* effects of nerve growth factor on central cholinergic neurons, *Proc. Natl. Acad. Sci. USA* 86 (1989) 2056–2060.
- [2] Cuello A.C., Thoenen H., The Pharmacology of Neurotrophic Factors, in: Claudio Cuello A., Collier B. (Eds.), *Pharmacological Sciences: Perspectives for Research and Therapy in the Late 1990s*, Birkhauser Verlag, Basel, 1995, pp. 241–254.
- [3] Debeir T., Benavides J., Vige X., Dual effects of thrombin and a 14-amino acid peptide agonist of the thrombin receptor on septal cholinergic neurons, *Brain Res.* 708 (1996) 159–166.
- [4] Dreyfus C.F., Bernd, P., Martinez, H.J., Rubin, S.J. and Black, I.B., GABAergic and cholinergic neurons exhibit high affinity nerve growth factor binding in the rat basal forebrain, *Exp. Neurol.* 104 (1989) 181–185.
- [5] Fonnum F., A rapid radiochemical method for the determination of choline acetyltransferase, *J. Neurochem.* 24 (1975) 407–409.
- [6] Garofalo L., Ribeiro-da-Silva A., Cuello A.C., Nerve growth factor-induced synaptogenesis and hypertrophy of cortical cholinergic terminals, *Proc. Natl. Acad. Sci. USA* 89 (1992) 2639–2643.
- [7] Garofalo L., Ribeiro-da-Silva A., Cuello A.C., Potentiation of nerve growth factor-induced alterations in cholinergic fibre length and presynaptic terminal size in cortex of lesioned rats by the monosialoganglioside GM1, *Neuroscience* 57 (1993) 21–40.

- [8] Gnahn H., Hefti F., Heumann R., Schwab M.E., Thoenen H., NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: Evidence for a physiological role of NGF in the brain?, *Dev. Brain Res.* 9 (1983) 45–52.
- [9] Hatanaka H., Nihonmatsu I., Tsukui H., Nerve growth factor promotes survival of cultured magnocellular neurons from nucleus basalis of Meynert in postnatal rats, *Neurosci. Lett.* 90 (1988) 63–68.
- [10] Johnston H.V., Rutkowski J.L., Wainer B.H., Long J.B., Mobley W.C., NGF effects on developing forebrain cholinergic neurons are regionally specific, *Neurochem. Res.* 12 (1987) 985–994.
- [11] LeSauter L., Wei L., Gibbs B.F., Saragovi H.U., Small peptide mimics of nerve growth factor bind TrkA receptors and affect biological responses *J. Biol. Chem.* 270 (1995) 6564–6569.
- [12] LeSauter L., Cheung N.K.V., Lisbona R., Saragovi H.U., Small molecule nerve growth factor analogs image receptors in vivo, *Nature BioTech.* 14 (1996) 1120–1122.
- [13] Lewin G.R., Barde Y.A., Physiology of the neurotrophins, *Annu. Rev. Neurosci.* 19 (1996) 289–317.
- [14] Lindsay R.M., Wiegand S.J., Altar C.A., DiStefano P.S., Neurotrophic factors: From molecule to man, *Trends Neurosci.* 17 (1994) 182–190.
- [15] Paxinos G., Watson C., The rat brain in stereotaxic coordinates, Academic Press, Sydney, Australia, 1986.
- [16] Thoenen H., The changing scene of neurotrophic factors, *Trends Neurosci.* 14 (1991) 165–170.
- [17] Wong T.P., Campbell P.M., Ribeiro-da-silva A., Cuello A.C. Synaptic numbers across cortical laminae and cognitive performance of the rat during ageing, *Neuroscience* 84 (1998) 403–412.

Nicotine modifies the activity of ventral tegmental area dopaminergic neurons and hippocampal GABAergic neurons

Janet L. Fisher, Volodymyr I. Pidoplichko, John A. Dani*

Division of Neuroscience, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030-3498, USA

Abstract — While trying to mimic the dose and time course of nicotine as it is obtained by a smoker, we found the following results. The initial arrival of even a low concentration of nicotine increased the firing rate of dopaminergic neurons from the ventral tegmental area (VTA) and increased the spontaneous vesicular release of GABA from hippocampal neurons. Longer exposure to nicotine caused variable, but dramatic, desensitization of nicotinic receptors and diminished the effects of nicotine. The addictive properties of nicotine as well as its diverse effects on cognitive function could be mediated through differences in activation and desensitization of nicotinic receptors in various areas of the brain. (©Elsevier, Paris)

Résumé — La nicotine modifie l'activité des neurones dopaminergiques de l'aire tegmentale ventrale et des neurones gabaergiques de l'hippocampe. L'arrivée initiale de même une faible concentration de nicotine augmente la décharge des neurones dopaminergiques de l'aire tegmentale ventrale (VTA) et augmente la libération vésiculaire spontanée de GABA par les neurones de l'hippocampe. Une exposition prolongée à la nicotine cause une désensibilisation des récepteurs nicotiniques variable mais prononcée et diminue les effets de la nicotine. (©Elsevier, Paris)

nicotine / dopamine / GABA / VTA / hippocampus

1. Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) were originally classified into two broad groups, the α -bungarotoxin (α -BTX)-binding sites and the more standard high-affinity nicotine binding sites [7, 18, 27, 28]. The nAChR subunits expressed in neurons include eight α (α 2– α 9) and three β (β 2– β 4) subunits. In heterologous expression systems, most subunits associate in combinations of α and β subunits to form functional nAChRs. The α 7, α 8, and α 9 subunits are capable of forming homomeric receptors. In mammalian brain most of the α -BTX-binding sites are probably synonymous with α 7-containing nAChRs.

Although the endogenous transmitter for nAChRs is acetylcholine, these receptors are also a site of action for nicotine obtained exogenously through tobacco. Smoking tobacco can deliver a small pulse of nicotine associated with each cigarette that is superimposed on a lower steady-state nicotine level maintained during the day. Nicotine is generally acknowledged as the addictive component of cigarettes [11, 29]. In addition, nicotine has numerous effects on cognitive function that may be mediated through nAChRs [17].

Ventral tegmental area (VTA) neurons of the mesolimbic dopamine system are thought to play an integral role in reinforcing rewarding behaviors. Drugs of addiction (such as cocaine, amphetamine, and nicotine) can exert influence over the mesolimbic system. Many addictive drugs increase dopamine release in the nucleus accumbens, which receives dopaminergic input from the VTA [24]. In vivo studies have shown that nicotine self-administration is reduced by lesions of the mesolimbic pathway or by nicotinic antagonists microinfused into the VTA [8, 9]. VTA neurons express nAChRs and can be stimulated to fire action potentials by nicotine [4–6, 23].

Using addictive drugs accrues learned behaviors, and contextual cues can elicit cravings long after drug use has ceased [17]. The hippocampus is an important center for contextual and spatial learning that receives cholinergic innervation and richly expresses nAChRs [13, 31]. GABAergic inhibitory interneurons in the hippocampus express nAChRs and nicotinic currents [2, 12, 14]. Nicotine has been shown to increase GABA release from several types of neurons from mouse, rat, and chick [3, 15, 16, 19] (for review see [30]). In CA1 hippocampal neurons, high concentrations of nicotine were found to increase spontaneous GABA release [2].

In both the VTA and the hippocampus, there is little information regarding the effects of sustained low concentrations of nicotine, as would be typically achieved by smokers. Therefore, we examined the

* Correspondence and reprints

ability of low concentrations of nicotine to modify the activity of the dopaminergic VTA neurons and GABAergic hippocampal neurons.

2. Materials and methods

2.1. Ventral tegmental slice preparation

Slices were prepared from Sprague-Dawley rats (12–25 days) using standard techniques as previously described [23]. The external bath solution contained (in mM): 135 NaCl, 2.5 KCl, 2.5 CaCl₂, 1 MgCl₂, 21 NaHCO₃, and 10 dextrose. The bath solution was continuously flowing and was bubbled with 95% O₂ and 5% CO₂. In most cases atropine (0.25 or 1 μ M) was present to inhibit muscarinic acetylcholine receptors. The patch pipettes were filled with an internal solution containing (in mM): 120 CsCH₃SO₃, 10 CsCl, 10 EGTA, 5 Mg-ATP, 0.3 Na₃GTP, 10 HEPES, with pH 7.3. For current-clamp recordings half of the CsCH₃SO₃ was replaced by KCH₃SO₃.

2.2. Hippocampal primary culture

Hippocampal cell cultures were prepared from 1–3-day-old Sprague-Dawley rats as previously described [32]. Dissociated neurons were plated onto coverslips coated to have microislands of poly-D-lysine and collagen. The neurons were used for experiments after 15–30 days in culture. The external solution was composed of (in mM): 150 NaCl, 2.5 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, 0.5–1 μ M TTX, 10 μ M CNQX, with pH 7.3. The internal solution contained (in mM): 150 CsCH₃SO₃, 5 NaCl, 0.2 EGTA, 2–5 MgATP, 0–2 Na₂ATP, 0–0.3 Na₃GTP, 10 HEPES, with pH 7.3. Standard patch-clamp techniques were used.

3. Results

3.1. Effect of nicotine on the activity of dopaminergic VTA neurons

Dopaminergic neurons in the VTA were identified by two characteristics. In slice preparations, these neurons exhibit an inward current (I_h) in response to hyperpolarizing steps (*figure 1A*) [20]. In addition, some neurons were back-filled with biocytin, then positively labeled with antibodies for tyrosine hydroxylase, an enzyme required for dopamine synthesis. During current clamp recording under control conditions, spontaneous action potentials were observed. Bath application of 0.1 or 0.5 μ M nicotine caused a depolarization of the neuron and an increase in the frequency of spontaneous action potentials (*figure 1B*). On average, 0.1 μ M nicotine produced a depolarization of 4.3 ± 0.2 mV ($n = 6$) while 0.5 μ M nicotine caused a larger depolarization of 8 ± 1 mV ($n = 12$). The depolarization was due to an inward current in response to nicotine that was ob-

served in voltage-clamp recordings [23]. The majority of the nicotinic currents were inhibited by mecamylamine (5 μ M), which at low concentrations is selective for the standard nAChRs. A smaller component of the current in a minority of neurons was inhibited by methyllycaconitine (10 nM), which selectively inhibits α -BTX-sensitive, $\alpha 7$ -containing nAChRs.

Because nicotine obtained from tobacco has a long half-life, we examined the effect of a longer exposure to low concentrations of nicotine. The membrane voltage of a VTA neuron was monitored in whole-cell mode, while brief injections of 1 mM ACh were applied locally to a small portion of the soma (*figure 1C*). The local injection of ACh caused a depolarization that induced a burst of 3–5 action potentials. Then, a low concentration of nicotine (0.5 μ M) was applied to the bath. In the continued presence of nicotine, the response to ACh decreased. The neuron began to fire action potentials continuously and, then, the neuron became silent even during the local ACh injection. After washing for > 10 min, the ACh injection again could elicit action potentials. It is likely that the nAChRs became desensitized by the low concentration of nicotine and, consequently, were unable to respond to the ACh injections. These results suggest that the exposure to nicotine observed after smoking a cigarette could serve to boost activity initially. Subsequently, however, long exposure to nicotine could reduce the responsiveness of these VTA neurons to nicotine and to endogenous cholinergic afferent input.

3.2. Effect of nicotine on spontaneous vesicular release of GABA from hippocampal neurons

Spontaneous GABA miniature inhibitory postsynaptic currents (mIPSCs) were observed in cultured hippocampal neurons. Microisland cultures were used so that synaptic connections could be made only among neurons on that same island [25]. This allowed applications of nicotine to a single island to reach all the neurons that might form connections with the voltage-clamped neuron. Microislands used for these experiments typically contained 2–5 neurons. Inhibitors of ionotropic glutamate receptors (10 μ M CNQX) and voltage-gated Na⁺ channels (0.5 μ M TTX) were included to isolate the spontaneous GABA-mediated currents. At a holding potential of –20 mV, outward currents were commonly observed (*figure 2A*). Net Cl[–] movement was inward at this potential (giving an outward positive current) because the internal solution contained a relatively low concentration of Cl[–]. The mIPSCs had a relatively long duration (25–100 ms), typical of GABA_A receptor mediated synaptic currents. We have

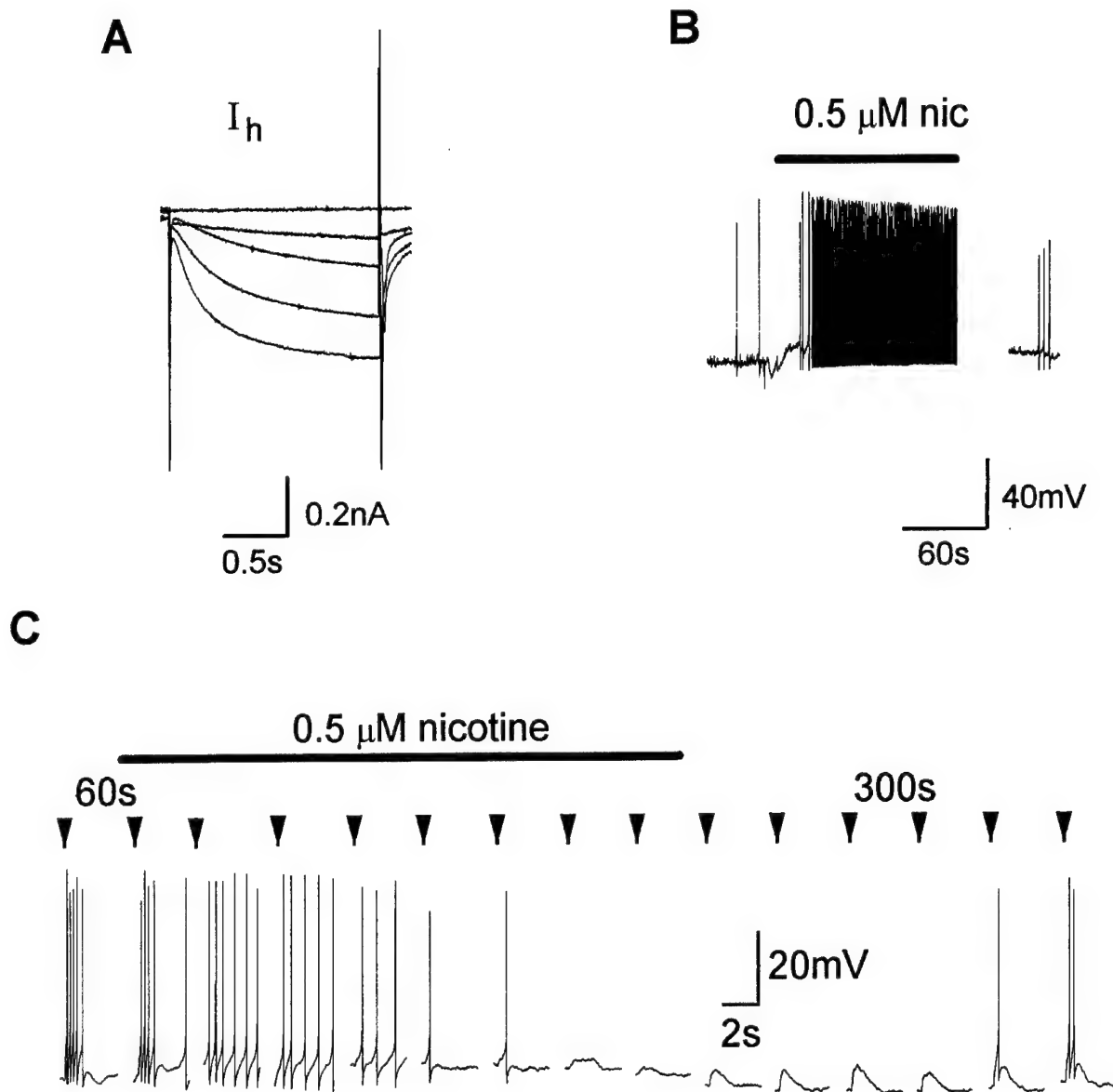


Figure 1. Nicotine initially increases the activity of dopaminergic VTA neurons, but desensitization reduces the effect of nicotine over time. **A.** Whole-cell voltage-clamped VTA neurons respond to hyperpolarizing voltage steps with an inward I_h current, characteristic of dopaminergic neurons. The neuron was held at -60 mV and negative 10 mV steps were applied (from -60 to -100 mV) to induce I_h . **B.** Bath application of $0.5 \mu\text{M}$ nicotine, as indicated by the solid bar, caused a depolarization and stimulated a burst of action potentials from a current-clamped VTA neuron. Following a 10 -min washout period, activity returned to baseline levels. **C.** During a longer bath application of $0.5 \mu\text{M}$ nicotine, multiple effects were observed. Local injection of 1 mM ACh for 40 ms (arrow heads) onto a small area of the soma of a current-clamped VTA neuron caused an initial depolarization that stimulated a small number of action potentials. Bath application of $0.5 \mu\text{M}$ nicotine (solid bar), gradually reduced the responsiveness to ACh and caused the neuron initially to fire a train of action potentials. Subsequently, the neuron stopped responding to the local ACh injections and the bath applied nicotine. The injections of ACh were given every 20 s, but the time interval between displayed traces is altered for clarity from 60 to 300 s. Figures adapted from Pidoplichko et al. [23]. Reprinted by permission from Nature, copyright 1997 Macmillan Magazines Ltd.

previously shown that these events are blocked by picrotoxin, confirming that they are mediated by GABA_A receptors [26].

The baseline frequency of mIPSCs was obtained by recording for several minutes before the application of nicotine. Only cells in which the frequency of events remained stable during this period were analyzed further. Nicotine (0.5 μ M) was applied to hippocampal neurons for 3–4 min., and the mIPSCs were recorded continuously during this period (figure 2). The frequency of events increased an average of 1.8 ± 0.2 -fold during the nicotine application ($P \leq 0.05$, paired t -test, $n = 3$). The frequency of events returned to the baseline level during or immediately following the nicotine application period. In some cases, after washing for a few minutes, another application of nicotine could again increase the frequency of spontaneous events. Although the frequency of the events increased, their amplitudes were unchanged [26].

Before nicotine



During 0.5 μ M nicotine

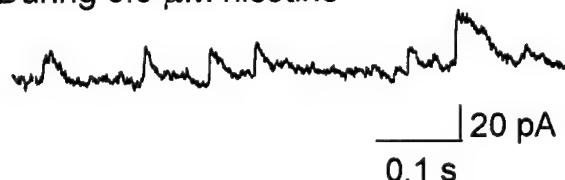


Figure 2. Nicotine increased the frequency of mIPSCs, but did not affect the amplitude. Representative traces of mIPSCs before and during bath application of 0.5 μ M nicotine. Hippocampal neurons in primary culture were voltage-clamped and held at -20 mV. Due to the low concentration of intracellular Cl^- , opening of GABA_A receptor channels results in a net outward current.

4. Discussion

Nicotine has a wide range of psychopharmacological effects that may be mediated by nAChRs throughout the CNS [11]. The low concentrations of nicotine achieved by smokers could alter the activity of neurons in many of the diverse regions of the brain that express nAChRs. Our results show that low concentrations of nicotine can influence synaptic

activity in both the hippocampus and the VTA. Nicotine increased the firing frequency of dopaminergic neurons from the VTA and increased spontaneous synaptic activity of GABAergic hippocampal neurons.

Current evidence suggests that the dopaminergic release from the VTA is increased by addictive drugs (including nicotine), and that the increased activity of these neurons is associated with reinforcement of the addictive behavior [9, 10, 24]. Our results show that nicotine initially increases the activity of VTA neurons even at the low nicotine concentrations commonly achieved by smokers. The pharmacological profile of the response to nicotine suggested that $\alpha 4 \beta 2$ receptors represent most of the nAChRs in the VTA, with a smaller component of $\alpha 7$ -containing receptors [23]. It has recently been shown that mice that do not express the $\beta 2$ subunit are less susceptible to addictive properties of nicotine [22]. Unlike the short-lived exposure to high concentrations of agonist that would be expected from activity of a cholinergic neuron, the nicotine levels of smokers remain at detectable levels for over 2 h. Our results indicate that longer exposure to nicotine causes desensitization and diminishes the stimulatory effect on the VTA neurons.

Nicotine also produces a variety of effects on cognitive function, including changes in learning and memory that may be mediated through nAChRs in the hippocampus. We have shown previously that nicotine acts on $\alpha 7$ -containing nAChR and directly increases the presynaptic concentration of Ca^{2+} , which in turn enhances the release of glutamate [13, 25]. The nicotine-induced Ca^{2+} influx was monitored with Fura-2 from single mossy-fiber presynaptic terminals in hippocampal slice while inhibiting glutamate, Na^+ , and Ca^{2+} channels [13].

Our results with GABAergic neurons are similar to those reported for glutamatergic hippocampal neurons. Nicotine increased mIPSC frequency without affecting the amplitude of the events. The response to nicotine occurred in the presence of TTX, suggesting a presynaptic site of action for nicotine in these neurons. Based on related work, the effects of nicotine are predominantly mediated by $\alpha 7$ -containing receptors [13, 25, 26]. This finding is consistent with evidence that nAChRs in the hippocampus are predominantly of the $\alpha 7$ -containing type [1, 21, 33]. Regulation of the activity of both glutamatergic and GABAergic neurons in the hippocampus by nicotine suggests that cholinergic inputs have the potential to tightly regulate synaptic transmission in the hippocampus, providing mechanisms for modulation of both excitatory and inhibitory systems.

References

- [1] Alkondon M., Albuquerque E.X., Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. I. Pharmacological and functional evidence for distinct structural subtypes, *J. Pharmacol. Exp. Ther.* 265 (1993) 1455–1473.
- [2] Alkondon M., Pereira E.F., Barbosa C.T., Albuquerque E.X., Neuronal nicotinic acetylcholine receptor activation modulates gamma-aminobutyric acid release from CA1 neurons of rat hippocampal slices, *J. Pharmacol. Exp. Ther.* 283 (1997) 1396–1411.
- [3] Bertolino M., Kellar K.J., Vicini S., Gillis R.A., Nicotinic receptor mediates spontaneous GABA release in the rat dorsal motor nucleus of the vagus, *Neuroscience* 79 (1997) 671–681.
- [4] Brodie M.S., Low concentrations of nicotine increase the firing rate of neurons of the rat ventral tegmental area in vitro, in: Adlkofer F., Thurau K. (Eds.), *Advances in Pharmacological Sciences. Effects of Nicotine on Biological Systems*, Birkhauser Verlag, Basel, 1991, 373–377.
- [5] Calabresi P., Lacey M.G., North R.A., Nicotinic excitation of rat ventral tegmental neurones in vitro studied by intracellular recording, *Br. J. Pharmacol.* 98 (1989) 135–140.
- [6] Clarke P.B., Nicotinic receptors in mammalian brain: localization and relation to cholinergic innervation, *Progr. Brain Res.* 98,77–83 (1993) 77–83.
- [7] Colquhoun L.M., Patrick J.W., Pharmacology of neuronal nicotinic acetylcholine receptor subtypes, *Adv. Pharmacol.* 39 (1997) 191–220.
- [8] Corrigan W.A., Coen K.M., Selective dopamine antagonists reduce nicotine self-administration, *Psychopharmacology (Berl.)* 104 (1991) 171–176.
- [9] Corrigan W.A., Franklin K.B., Coen K.M., Clarke P.B., The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine, *Psychopharmacology (Berl.)* 107 (1992) 285–289.
- [10] Corrigan W.A., Coen K.M., Adamson K.L., Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area, *Brain Res.* 653 (1994) 278–284.
- [11] Dani J.A., Heinemann S., Molecular and cellular aspects of nicotine abuse, *Neuron* 16 (1996) 905–908.
- [12] Frazier C.J., Rollins Y.D., Breese C.R., Leonard S., Freedman R., Dunwiddie T.V., Acetylcholine activates an α -Bungarotoxin-sensitive nicotinic current in rat hippocampal interneurons, but not pyramidal cells, *J. Neurosci.* 18 (1998) 1187–1195.
- [13] Gray R., Rajan A.S., Radcliffe K.A., Yakehiro M., Dani J.A., Hippocampal synaptic transmission enhanced by low concentrations of nicotine, *Nature* 383 (1996) 713–716.
- [14] Jones S., Yakel J.L., Functional nicotinic ACh receptors on interneurons in the rat hippocampus, *J. Physiol. (Lond.)* 504 (1997) 603–610.
- [15] Lena C., Changeux J.P., Pathological mutations of nicotinic receptors and nicotine-based therapies for brain disorders, *Curr. Opin. Neurobiol.* 7 (1997) 674–682.
- [16] Lena C., Changeux J.P., Mulle C., Evidence for 'preterminal' nicotinic receptors on GABAergic axons in the rat interpeduncular nucleus, *J. Neurosci.* 13 (1993) 2680–2688.
- [17] Levin E.D., Briggs S.J., Christopher N.C., Rose J.E., Persistence of chronic nicotine-induced cognitive facilitation, *Behav. Neural. Biol.* 58 (1992) 152–158.
- [18] McGehee D.S., Role L.W., Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons, *Annu. Rev. Physiol.* 57 (1995) 521–546.
- [19] McMahon L.L., Yoon K.W., Chiappinelli V.A., Nicotinic receptor activation facilitates GABAergic neurotransmission in the avian lateral spiriform nucleus, *Neuroscience* 59 (1994) 689–698.
- [20] Mercuri N.B., Bonci A., Calabresi P., Stefani A., Bernardi G., Properties of the hyperpolarization-activated cation current I_h in rat midbrain dopaminergic neurons, *Eur. J. Neurosci.* 7 (1995) 462–469.
- [21] Orr-Urtreger A., Göldner F.M., Saeki M., Lorenzo I., Goldberg L., De Biasi M., Dani J.A., Patrick J.W., Beauge L., Mice deficient in the $\alpha 7$ neuronal nicotinic receptor lack α -Bungarotoxin binding sites and hippocampal fast nicotinic currents, *J. Neurosci.* 17 (1997) 9165–9171.
- [22] Picciotto M.R., Zoli M., Rimondini R., Lena C., Marubio L.M., Pich E.M., Fuxe K., Changeux J.P., Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine, *Nature* 391 (1998) 173–177.
- [23] Pidoplichko V.I., DeBiasi M., Williams J.T., Dani J.A., Nicotine activates and desensitizes midbrain dopamine neurons, *Nature* 390 (1997) 401–404.
- [24] Pontieri F.E., Tanda G., Orzi F., Di Chiara G., Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs, *Nature* 382 (1996) 255–257.
- [25] Radcliffe K., Dani J.A., Nicotinic stimulation produces multiple forms of glutamatergic synaptic enhancement, *J. Neurosci.* (1998), in press.
- [26] Radcliffe K., Fisher J.L., Gray R., Dani J.A., Nicotinic modulation of glutamate and GABA synaptic transmission in hippocampal neurons, *Ann. N.Y. Acad. Sci.* (1998), in press.
- [27] Role L.W., Berg D.K., Nicotinic receptors in the development and modulation of CNS synapses, *Neuron* 16 (1996) 1077–1085.
- [28] Sargent P.B., The diversity of neuronal nicotinic acetylcholine receptors, *Annu. Rev. Neurosci.* 16 (1993) 403–443.
- [29] Stolerman I.P., Shoaib M., The neurobiology of tobacco addiction, *Trends Pharmacol. Sci.* 12 (1991) 467–473.
- [30] Wonnacott S., Presynaptic nicotinic ACh receptors, *Trends Neurosci.* 20 (1997) 92–98.
- [31] Woolf N.J., Cholinergic systems in mammalian brain and spinal cord, *Progr. Neurobiol.* 37 (1991) 475–524.
- [32] Zarei M.M., Dani J.A., Structural basis for explaining open-channel blockade of the NMDA receptor, *J. Neurosci.* 15 (1995) 1446–1454.
- [33] Zarei M., Radcliffe K.A., Chen D., Patrick J.W., Dani J.A., Distribution of nicotinic acetylcholine $\alpha 7$ and $\beta 2$ subunits on cultured hippocampal neurons, *Neuroscience* (1998), in press.

The hypothesis of an ambient level of acetylcholine in the central nervous system

Laurent Descarries

Départements de pathologie et biologie cellulaire et de physiologie, and Centre de recherche en sciences neurologiques, Faculté de médecine, Université de Montréal, C.P. 6128, Succ. Centre-ville, Montreal, Québec, Canada H3C 3J7

Abstract — Recent ultrastructural data demonstrate the largely asynaptic character of the cholinergic innervation in many regions of adult rat brain. These data favour the hypothesis of a diffuse transmission/modulation by acetylcholine in the CNS and, by way of consequence, that of a persistent, low level of acetylcholine in the extracellular space. (©Elsevier, Paris)

Résumé — L'hypothèse d'un niveau ambiant d'acetylcholine dans le système nerveux central. Des données ultrastructurales récentes démontrent le caractère largement asynaptique de l'innervation cholinergique de plusieurs régions du cerveau chez le rat adulte. Ces données favorisent l'hypothèse d'une transmission/modulation diffuse par l'acetylcholine et, par voie de conséquence, celle de la persistance d'une faible concentration d'acetylcholine dans l'espace extracellulaire. (©Elsevier, Paris)

axon terminals / innervation / ultrastructure / diffuse transmission / acetylcholinesterase

1. Introduction

Much of the current knowledge about the properties and function of acetylcholine (ACh) in mammalian brain has been acquired before it was even possible to identify the neurons which synthesize and release this molecule as transmitter/modulator. Already in the sixties, biochemical and electrophysiological studies had revealed that ACh was implicated in cortical activation [25, 33] and sleep and wakefulness [5, 23, 24]. Subsequently, ACh was shown to play a role in learning [48], memory [4, 10] and cortical plasticity [3]. In the early eighties, specific antibodies were produced against the biosynthetic enzyme of ACh, choline acetyltransferase (ChAT), allowing for the immunocytochemical identification of these neurons [9, 17, 28]. Until the nineties, however, the light and electron microscopic demonstration of central ACh axon terminals (varicosities) remained difficult (reviewed in [22]), presumably because of a relatively low affinity of available antibodies. It is only in 1989 that Costantino Cozzari, working with Boyd K. Hartman in Minneapolis, raised and characterized a highly sensitive monoclonal antibody directed against purified rat ChAT [8], which we were privileged to use for examining the ultrastructural features of these terminals in rat brain.

Some of these results are briefly reviewed here, as a basis for re-expounding a recently proposed hypothesis according to which the spontaneous and evoked release of ACh from predominantly asynaptic axon terminals might permanently maintain a basal level of ACh in the extracellular space, at least in

brain regions densely innervated by ACh neurons [7, 11, 13, 14].

2. Cerebral cortex

The ACh innervation of rat cerebral cortex (*figure 1A*) arises mainly from the nucleus basalis magnocellularis (also designated as substantia innominata, nucleus basalis of Meynert, or group Ch4) [27, 32]. The density of this innervation appears to be somewhat higher than that of its serotonin counterpart, estimated to be in the order of 5.8×10^6 varicosities per mm^3 [1]. In rodents, intracortical neurons [18, 26, 29] would account for approximately 20% of these terminals [19].

In an extensive electron microscopic investigation [44], we examined the intrinsic and relational features of ChAT-immunostained axon varicosities in the primary somatosensory area (S1 or Par 1) of adult rat cortex. 812 terminals from the different cortical layers were scrutinized in long, uninterrupted series of thin sections across their whole volume. Several were actually reconstructed in three dimensions (*figure 1B*); about 200 were visualized as doublets or triplets of varicosities along the same axon.

A totally unexpected finding was that, in every layer of the cortex, only a low proportion of these ACh varicosities displayed a synaptic membrane differentiation (junctional complex). As indicated in *table I*, their mean synaptic incidence across layers was 14%. Only layer V showed a slightly higher proportion of synaptic varicosities (21%). As a whole, cortical ACh varicosities were relatively small,

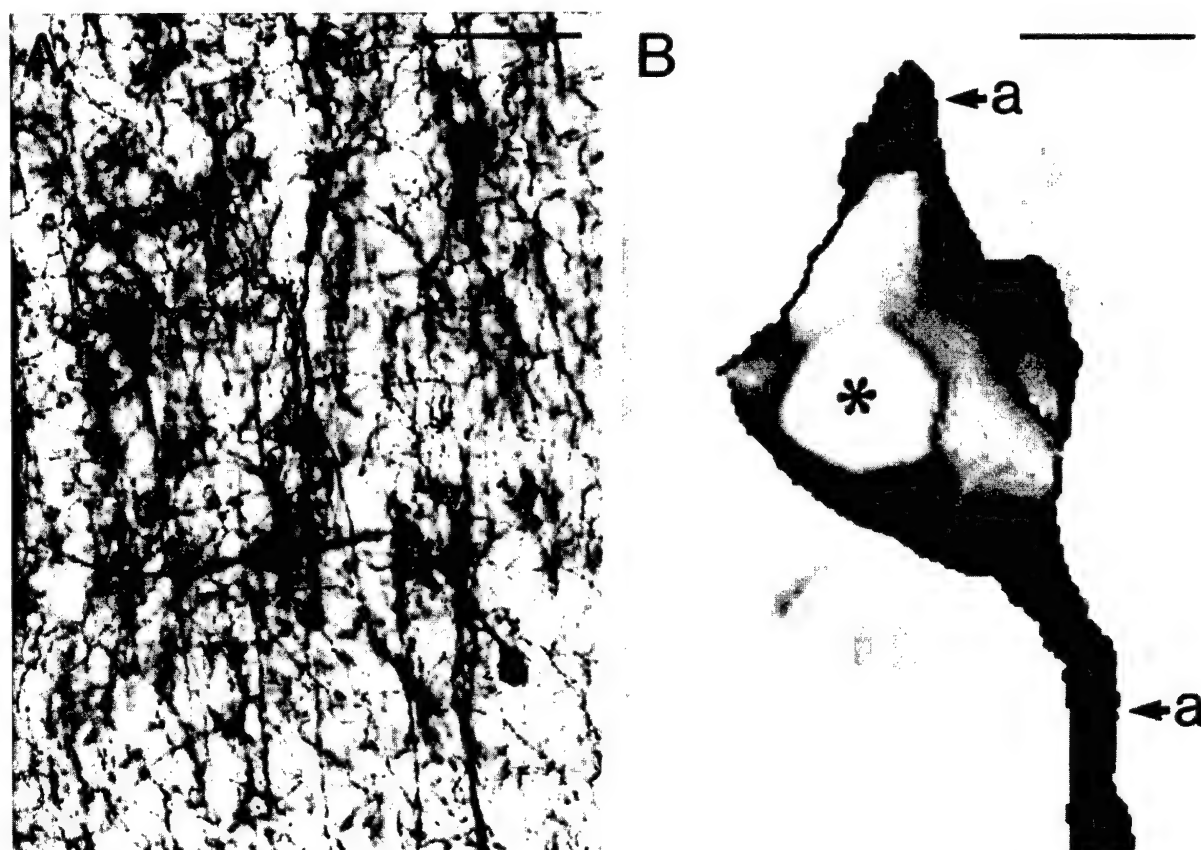


Figure 1. **A.** Light microscopic overview of the ChAT-immunostained innervation in layers II-III of adult rat parietal cortex (Par 1). Four immunoreactive cell bodies are visible in this field. The center one exhibits the typical features of smooth dendrite, fusiform ACh interneurons. The intricate network of thin varicose fibers pervading the neuropil arises in large part from the nucleus basalis magnocellularis. Note the irregular course of these axonal branches, which seemingly run in every direction, forming a relatively tight criss-crossing pattern with no predilection for the immediate vicinity of neuronal somata or the microvessels. Numerous bifurcations are visible (e.g., arrowheads). Smaller and larger varicosities may be observed on the same axons. Magnification $\times 450$. Scale bar, 50 μm . **B.** Three dimensional electron microscopic reconstruction of a ChAT-immunostained axonal varicosity from layer I of the parietal cortex. This varicosity, visualized along its parent axon (a), was examined in its entirety in a series of 11 ultrathin sections (see figure 8 in [44]). It was reconstructed with the Volume Analysis Station ICAR 80.8 (ISG Technologies) from Nissei Sangyo Canada. A reference section from the middle of the series was included as background for purposes of orientation. The whole surface of the varicosity was observed and there was no hint of a synaptic specialization. The large, smooth protrusion directed toward the observer (*) marked the presence of an underlying mitochondrion. Magnification $\times 46\,000$. Scale bar, 0.5 μm (reproduced with permission from [44]).

Table I. Comparative data on the synaptic incidence of ACh axon terminals in adult rat CNS.

	ACh (%)	NA (%)	5-HT (%)	GABA (%)
Cerebral cortex (Par 1)	14	17	46	—
Hippocampus (CA1)	7	16	23	108
Neostriatum	9	—	13	—

From Umbriaco et al. [44] and Séguéla et al. [40, 41] for the cerebral cortex, Umbriaco et al. [45] for the hippocampus, and Contant et al. [7] and Soghomonian et al. [43] for the neostriatum.

averaging 0.6 μm in diameter. In general, those bearing a synaptic junction were slightly larger than their non-synaptic counterparts, but both junctional and non-junctional varicosities could be observed on the same axons. The junctional complexes formed by cortical ACh terminals were single, almost always symmetrical, and occupied a small fraction of the total surface of varicosities ($< 3\%$). These synaptic varicosities usually contacted dendritic branches (76%), less often spines (24%), and none were seen on cell bodies.

The low synaptic incidence of cortical ACh varicosities in rat was confirmed in two subsequent investigations focused on the neurovascular relationships of these terminals [6, 46]. Both reports emphasized the fact that ChAT-immunostained boutons in the immediate vicinity of capillaries or microarterioles seldom exhibited synaptic specializations. The synaptic incidence of these perivascular ACh varicosities was then shown to be low not only in the frontoparietal (14%), but also in the perirhinal cortex (9%).

None of the previous ChAT immunocytochemical studies had actually been devised to determine the synaptic incidence of ACh terminals. In 1995, however, Mrzljak et al. [35] reported on 100 serially sectioned ChAT immunoreactive boutons at the border of layers II and III in macaque monkey prefrontal cortex. Only 44% of these varicosities were found to make synapse, 56% being without any visible junctional specialization, even if frequently juxtaposed to dendrites or spines receiving asymmetrical synapses. Very recently, Smiley et al. [42] performed a similar study on two samples of human anterior temporal lobe removed at surgery. They found 67% of 42 varicosities from layers I and II endowed with small but identifiable synaptic specializations. Whether such differences from the rat reflect sampling biases, regional differences or species differences remains to be determined. In all events, the results allow the inference that, in primates as well as rat cortex, ACh may subserve at least two types of modulatory effects: some dependent on the existence of symmetrical synaptic junctions, mostly made with dendritic shafts and spines; the others exerted on neuronal targets merely apposed to asynaptic ACh terminals, or even more remote from these presumed release sites.

3. Hippocampus

The ACh innervation of hippocampus originates mainly from cell bodies located in groups Ch1 and Ch2, ie, in the medial septal nucleus and nucleus of the vertical limb of the diagonal band of Broca [31, 39]. The density of this ACh innervation is again similar to that of its serotonin counterpart, estimated to average 2.7×10^6 varicosities per mm^3 [38]. In this region, the main purpose of our investigation was to compare the ACh terminals with three other types of endings also defined by their content in transmitter: noradrenaline (NA), serotonin (5-HT) and gamma-aminobutyric acid (GABA), as respectively immunostained with antibodies against NA, 5-HT, and glutamic acid decarboxylase [45]. The four types of varicosities were examined in the same

portion of the stratum radiatum of CA1. The synaptic incidence was estimated from single thin sections with the stereological formula of Beaudet and Sotelo [2], the reliability of which had been experimentally verified in the serial section study of cortical terminals [44].

As shown in *table I*, the hippocampal ACh varicosities were even more asynaptic than their NA and 5-HT counterparts, in contrast to the GABA terminals which were all synaptic. The extrapolated synaptic incidence for the GABA endings exceeded 100% because of the occasional occurrence of more than one junction per varicosity.

4. Neostriatum

The ACh innervation of neostriatum is entirely issued from a fraction of the large interneurons which represent less than 2% of the total neuron population of this region [30, 49]. Yet, the ChAT immunoreactivity in neostriatum is the strongest in brain. Prior to our 1996 study [7], one could only imagine how profuse an axonal network might account for such labeling. This became immediately apparent when observing the multitude of small varicosities which pervaded the neostriatal neuropil. These were particularly small, as their mean diameter ($0.43 \mu\text{m}$) was significantly less than that of their cortical congeners and of striatal dopamine or serotonin varicosities (all averaging about $0.6 \mu\text{m}$ in diameter).

Once again, a vast majority of these ACh varicosities were asynaptic. In single thin section, only 2.7% exhibited a junctional complex compared with 57% of unlabeled varicosity profiles selected at random from the same micrographs. The stereological extrapolation to the whole volume of ACh varicosities indicated a real synaptic incidence of less than 10% (*table I*), whereas that for the unlabeled varicosities was much higher than 100% because of the frequent occurrence of multiple junctions. Direct appositions between immunostained varicosities were not uncommon, reflecting the extreme density of this ACh innervation. Striatal ACh varicosities were often juxtaposed to unlabeled varicosities, many of which were synaptic.

5. An ambient level of ACh in brain

As initially postulated in the case of monoamine terminals in the cerebral cortex (for review, see [12]), the lack of synaptic junction on a large proportion of ACh axon varicosities in adult rat cerebral cortex, hippocampus and neostriatum is suggestive of a diffuse mode of transmission, complementary

to classical synaptic transmission. Such transmission by ACh will account for direct axo-axonic effects in the absence of axo-axonic synapses, for the presence of specific receptors located on somatodendritic membrane away from synaptic junctions, and for prolonged modulatory effects onto neurons, glial cells and microvessels. In this regard, it is noteworthy that a recent immunoelectronmicroscopic study by Mrzljak et al. [34], using subtype-specific antibodies to m1 and m2 muscarinic receptors in the prefrontal cortex of macaque monkey cortex, has indeed demonstrated the frequent localization of these receptors on dendritic spines, dendritic branches and cell bodies, as well as non-homologous (i.e., non-ACh) axon varicosities, remote from ACh terminals.

In view of the observed density of the ACh innervation, particularly in neostriatum, a complementary aspect of the diffuse transmission paradigm must also be considered. According to this hypothesis, a low level of ambient ACh would be present throughout the extracellular space, at least in brain regions densely innervated by predominantly asynaptic ACh terminals. This ambient level would be permanently maintained by the spontaneous or evoked release from the majority of non junctional ACh varicosities and spillover from the minority that are junctional. The release events would result in local and transient fluctuations of this ambient level, representing superimposed signals over the preexisting information conveyed by ACh.

In this context, as recently discussed in detail [14], brain acetylcholinesterase (AChE), which predominantly consists of the tetrameric G4 molecular form, would primarily serve to maintain the ambient level of ACh within physiological limits, rather than totally eliminate ACh from synaptic clefts and the extracellular space. This would be in keeping with the notion that this same G4 form plays a similar role in skeletal muscle, where, in contrast to the A12 form, it is concentrated outside and around endplates [20], and therefore does not significantly contribute to the rapid removal of ACh from synaptic clefts. Actually, there is increasing evidence to suggest that the perijunctional G4 compartment deals with ACh molecules spilling over from (or diffusing out of) the synaptic cleft, thus preserving the excitability of endplates, notably during high frequency activity [15, 21].

In many parts of CNS, microdialysis data seems consistent with the existence of a steady-state, resting or ambient level of ACh in the extracellular space. Spontaneous dialysis outputs of ACh in the nanomolar range have been repeatedly measured in the cerebral cortex, hippocampus or neostriatum of freely moving rat, in the absence of AChE inhibitor (references in [14]). Preliminary results obtained

with the so-called zero net flux method also estimate this basal level to be in the nanomolar range [36]. Experimental and theoretical models have been proposed to evaluate the diffusion of transmitter molecules and answer the often-asked question of the distance that can be reached by these molecules in living brain [37]. In the case of dopamine, it has been inferred that a 10 μm distance might be reached within 50 ms [47]. Based on currently available estimates of the density of dopamine innervation in striatum [16], and considering that the density of striatal ACh innervation is at least equivalent, it may be calculated that a sphere of striatal neuropil of 10 μm radius contains about 400 dopamine and 400 ACh axon terminals, five to ten times more unidentified terminals, and at least several thousand dendritic spines. If ACh diffuses that far, this may explain why there would be such a variety of receptors for this single transmitter, and presumably different subtypes on any given neuron, perhaps for detecting different concentrations as informative signals.

A low ambient level of ACh could serve to regulate the expression and/or functional state of high-affinity receptor subtypes located on ACh (autoreceptors) or other neurons (heteroreceptors), glial cells and microvessels. Widely distributed homologous or heterologous receptors, activated by fluctuations of the basal level of ACh, could then be the ones to mediate complex behavioral effects produced by drugs acting on AChE or on the choline transporter. The existence of the ambient level could also explain the fact that certain pathological disorders attributed to loss of ACh neurons seem to become manifest only when a major proportion of these release sites have disappeared. Similarly, it might explain the beneficial effects of substitution, pharmacological or graft therapies, otherwise difficult to account for in the absence of restored synaptic connectivity.

Acknowledgments

This work was mainly supported by MRC grant MT-3544 from the Medical Research Council of Canada. The author is grateful to Victor Gisiger for a careful revision of the manuscript.

References

- [1] Audet M.A., Descarries L., Doucet G., Quantified regional and laminar distribution of the serotonin innervation in the anterior half of adult rat cerebral cortex, *J. Chem. Neuroanat.* 2 (1989) 29–44.

- [2] Beaudet A., Sotelo C., Synaptic remodeling of serotonin axon terminals in rat agranular cerebellum, *Brain Res.* 206 (1981) 305–329.
- [3] Bear M.F., Singer W., Modulation of visual cortical plasticity by acetylcholine and noradrenaline, *Nature* 320 (1986) 172–176.
- [4] Bowen D.M., Smith C.B., White P., Davison A.M., Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies, *Brain* 99 (1976) 459–496.
- [5] Celesia G.G., Jasper H.H., Acetylcholine released from cerebral cortex in relation to state of activation, *Neurology* 16 (1966) 1053–1063, 1070.
- [6] Chédotal A., Umbriaco D., Descarries L., Hartman B.K., Hamel E., Light and electron microscopic immunocytochemical analysis of the neurovascular relationships of choline acetyltransferase (ChAT) and vasoactive intestinal polypeptide (VIP) nerve terminals in the rat cerebral cortex, *J. Comp. Neurol.* 343 (1994) 57–71.
- [7] Contant C., Umbriaco D., Garcia S., Watkins K.C., Descarries L., Ultrastructural characterization of the acetylcholine innervation in adult rat neostriatum, *Neuroscience* 71 (1996) 937–947.
- [8] Cozzari C., Howard J., Hartman B., Analysis of epitopes on choline acetyltransferase (ChAT) using monoclonal antibodies (Mabs), *Soc. Neurosci. Abstr.* 16 (1990) 200.
- [9] Crawford G.D., Correa L., Salvaterra P.M., Interaction of monoclonal antibodies with mammalian choline acetyltransferase, *Proc. Natl. Acad. Sci. USA* 79 (1982) 7031–7035.
- [10] Davies P., Maloney A.J.F., Selective loss of central cholinergic neurons in Alzheimer's disease, *Lancet* II (1976) 1403.
- [11] Descarries L., Umbriaco D., Ultrastructural basis of monoamine and acetylcholine function in CNS, *Semin. Neurosci.* 7 (1995) 308–318.
- [12] Descarries L., Séguéla P., Watkins K.C., Nonjunctional relationships of monoamine axon terminals in the cerebral cortex of adult rat, in: Fuxe K., Agnati L.F. (Eds.), *Volume Transmission in the Brain: Novel Mechanisms for Neural Transmission*, Raven Press, New York, 1991, pp. 53–62.
- [13] Descarries L., Umbriaco D., Contant C., Watkins, K.C., A new hypothesis of acetylcholine (ACh) function in densely ACh-innervated regions of the brain, IVth IBRO World Congr. Neurosci. Abstr. A3.15, 1995, p. 101.
- [14] Descarries L., Gisiger V., Steriade, M., Diffuse transmission by acetylcholine in the CNS, *Progr. Neurobiol.* 53 (1997) 603–625.
- [15] Dörflöcher M., Irintchev A., Brinkers M., Wernig A., Effects of enhanced activity on synaptic transmission in mouse extensor digitorum longus muscle, *J. Physiol. (Lond.)* 436 (1991) 283–292.
- [16] Doucet G., Descarries L., Garcia S., Quantification of the dopamine innervation in adult rat neostriatum, *Neuroscience* 19 (1986) 427–445.
- [17] Eckenstein F., Thoenen H., Production of specific antisera and monoclonal antibodies to choline acetyltransferase: characterization and use for identification of cholinergic neurons, *EMBO J.* 1 (1982) 363–368.
- [18] Eckenstein F., Thoenen H., Cholinergic neurons in the rat cerebral cortex demonstrated by immunohistochemical localization of choline acetyltransferase, *Neurosci. Lett.* 36 (1983) 211–215.
- [19] Eckenstein F., Baughman R.W., Cholinergic innervation in cerebral cortex, in: Jones E.G., Peters A. (Eds.), *Cerebral Cortex. Further Aspects of Cortical Function, Including Hippocampus*, Vol. 6, Plenum Press, New York, 1987, pp. 129–160.
- [20] Gisiger V., Stephens H.R., Localization of the pool of G4 acetylcholinesterase characterizing fast muscles and its alteration in murine muscular dystrophy, *J. Neurosci. Res.* 19 (1988) 62–78.
- [21] Gisiger V., Bélisle M., Gardiner P.F., Acetylcholinesterase adaptation to voluntary wheel running is proportional to the volume of activity in fast, but not slow, rat hindlimb muscles, *Eur. J. Neurosci.* 6 (1994) 673–680.
- [22] Houser C.R., Cholinergic synapses in the central nervous system: studies of the immunocytochemical localization of choline acetyltransferase, *J. Electron Microsc. Tech.* 15 (1990) 2–19.
- [23] Jasper H.H., Tessier J., Acetylcholine liberation from cerebral cortex during paradoxical (REM) sleep, *Science* (1971) 601–602.
- [24] Kanai T., Szerb J.C., Mesencephalic reticular activating system and cortical acetylcholine output, *Nature* 205 (1965) 80–82.
- [25] Krnjević K., Phillis J.W., Acetylcholine-sensitive cells in the cerebral cortex, *J. Physiol. (Lond.)* 166 (1963) 296–327.
- [26] Lauterborn J.C., Isackson P.J., Montalvo R., Gall C.M., In situ hybridization localization of choline acetyltransferase mRNA in adult rat brain and spinal cord, *Mol. Brain Res.* 17 (1993) 59–69.
- [27] Lehmann J., Nagy J.I., Atmadia S., Fibiger H.C., The nucleus basalis magnocellularis: the origin of a cholinergic projection to the neocortex of the rat, *Neuroscience* 5 (1980) 1161–1174.
- [28] Levey A.I., Armstrong D.M., Atweh S.F., Terry R.D., Wainer B.H., Monoclonal antibodies to choline acetyltransferase: production, specificity, and immunohistochemistry, *J. Neurosci.* 3 (1983) 1–9.
- [29] Levey A.I., Wainer B.H., Rye D.B., Mufson E.J., Mesulam M.-M., Choline acetyltransferase-immunoreactive neurons intrinsic to rodent cortex and distinction from acetylcholinesterase-positive neurons, *Neuroscience* 13 (1984) 341–353.
- [30] McGeer P.L., McGeer E.G., Fibiger H.C., Wickson V., Neostriatal choline acetylase and cholinesterase following selective brain lesions, *Brain Res.* 35 (1971) 308–314.
- [31] McKinney M., Coyle J.T., Hedreen J.C., Topographic analysis of the innervation of the rat neocortex and hippocampus by the basal forebrain cholinergic system, *J. Comp. Neurol.* 217 (1983) 103–121.
- [32] Mesulam M.-M., Mufson E.J., Wainer B.H., Levey A.I., Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1–Ch6), *Neuroscience* 10 (1983) 1185–1201.
- [33] Mitchell J.F., The spontaneous and evoked release of acetylcholine from the cerebral cortex, *J. Physiol. (Lond.)* 165 (1963) 98–116.
- [34] Mrzljak L., Levey A.I., Goldman-Rakic P.S., Association of m1 and m2 muscarinic receptor proteins with asymmetric synapses in the primate cerebral cortex: morphological evidence for cholinergic modulation of excitatory neurotransmission, *Proc. Natl. Acad. Sci. USA* 90 (1993) 5194–5198.
- [35] Mrzljak L., Pappay M., Leranth C., Goldman-Rakic P.S., Cholinergic synaptic circuitry in the macaque prefrontal cortex, *J. Comp. Neurol.* 357 (1995) 603–617.
- [36] Newton P., Justice J.B. Jr, Quantitative microdialysis of acetylcholine under basal conditions and during acetylcholin-

- terase inhibition in the rat, Soc. Neurosci. Abstr. 21 (1995) 67.
- [37] Nicholson C., Syková E., Extracellular space structure revealed by diffusion analysis, Trends Neurosci. 21 (1998) 207–215.
 - [38] Oleskevich S., Descarries L., Quantified distribution of the serotonin innervation in adult rat hippocampus, Neuroscience 34 (1990) 19–33.
 - [39] Rye D.B., Wainer B.H., Mesulam M.-M., Mufson E.J., Saper C.B. (1984) Cortical projections arising from the basal forebrain: a study of cholinergic and non cholinergic components employing combined retrograde tracing and immunohistochemical localization of choline acetyltransferase, Neuroscience 13 (1984) 627–643.
 - [40] Séguéla P., Watkins K.C., Descarries, L., Ultrastructural relationships of serotonin axon terminals in the cerebral cortex of the adult rat, J. Comp. Neurol. 289, (1989) 129–142.
 - [41] Séguéla P., Watkins K.C., Geffard M., Descarries, L., Noradrenaline axon terminals in adult rat neocortex: an immunocytochemical analysis in serial thin sections, Neuroscience 35 (1990) 249–264.
 - [42] Smiley J.F., Morrell F., Mesulam, M.-M., Cholinergic synapses in human cerebral cortex: an ultrastructural study in serial sections, Exp. Neurol. (1997) 144, 361–368.
 - [43] Soghomonian J.-J., Descarries L., Watkins, K.C., Serotonin innervation in adult rat neostriatum. II. Ultrastructural features: a radioautographic and immunocytochemical study, Brain Res. 481 (1989) 67–86.
 - [44] Umbriaco D., Watkins K.C., Descarries L., Cozzari C., Hartman, B.K., Ultrastructural and morphometric features of the acetylcholine innervation in adult rat parietal cortex. An electron microscopic study in serial sections, J. Comp. Neurol. 348 (1994) 351–373.
 - [45] Umbriaco D., Garcia S., Beaulieu C., Descarries, L., Relational features of acetylcholine, noradrenaline, serotonin and GABA axon terminals in the stratum radiatum of adult rat hippocampus (CA1), Hippocampus 5 (1995) 605–620.
 - [46] Vaucher E., Hamel, E., Cholinergic basal forebrain neurons project to cortical microvessels in the rat: electron microscopic study with anterogradely transported *Phaseolus vulgaris* leucoagglutinin and choline acetyltransferase immunocytochemistry, J. Neurosci. 15 (1995) 7427–7441.
 - [47] Wightman R.M., Zimmerman J.B., Control of dopamine extracellular concentration in rat striatum by impulse flow and uptake, Brain Res. Rev. 15 (1990) 135–144.
 - [48] Woody C.D., Swartz B.E., Gruen E., Effects of acetylcholine and cyclic GMP on input resistance of cortical neurons in awake cats, Brain Res. 158 (1978) 373–395.
 - [49] Woolf N.J., Cholinergic systems in mammalian brain and spinal cord, Progr. Neurobiol. 37 (1991) 475–524.

The role of neuronal nicotinic acetylcholine receptors in antinociception: Effects of ABT-594

Michael W. Decker, Peter Curzon, Mark W. Holladay, Arthur L. Nikkel, R. Scott Bitner, Anthony W. Bannon, Diana L. Donnelly-Roberts, Pamela S. Puttfarcken, Theresa A. Kuntzweiler, Clark A. Briggs, Michael Williams, Stephen P. Arneric

*Neurological and Urological Diseases Research, Pharmaceutical Products Division, Abbott Laboratories, Dept. 47W
Building AP9A-LL, 100 Abbott Park Road, Abbott Park, IL 60064-3500, USA*

Abstract — ABT-594, a nicotinic acetylcholine receptor agonist, has antinociceptive effects in rat models of acute thermal, persistent chemical, and neuropathic pain. Direct injection of ABT-594 into the nucleus raphe magnus (NRM) is antinociceptive in a thermal threshold test and destruction of serotonergic neurons in the NRM attenuates the effect of systemic ABT-594. However, lidocaine-inactivation of the NRM prevents the antinociceptive effect of systemic (–)-nicotine but not that of systemic ABT-594. (©Elsevier, Paris)

Résumé — Le rôle des récepteurs nicotiniques neuronaux à l'acétylcholine dans l'antinociception. Effets de l'ABT-594. ABT-594, un agoniste du récepteur nicotinique à l'acétylcholine a des effets antinociceptifs dans des modèles de douleur induite par une température aiguë, un traitement chimique persistant et une neuropathie chez le rat. L'injection d'ABT-594 dans le grand noyau du Raphe (N.R.M.) est antinociceptif dans un test de sensibilité à la température et la destruction des noyaux sérotoninergiques atténue l'effet d'ABT-594. Cependant, l'inactivation du N.R.M. par la lidocaïne empêche l'effet antinociceptif de la nicotine mais pas de l'ABT-594. (©Elsevier, Paris)

ABT-594 / nucleus raphe magnus / nicotinic acetylcholine receptor / serotonin / antinociception

Most commonly used analgesics come from two classes: opioids and non-steroidal anti-inflammatory drugs (NSAIDs). Opioids are fully efficacious across a spectrum of pain states, but tolerance, side effects, and dependence issues are viewed as important limitations. NSAIDs, on the other hand, have more limited efficacy, being most useful in treating mild to moderate pain associated with inflammation, and have dose-limiting gastrointestinal effects.

Compounds acting at nicotinic acetylcholine receptors (nAChRs) also have antinociceptive activity, but have not been developed clinically. (–)-Nicotine, for example, is antinociceptive in rodents, but has low potency and efficacy and a short duration of action [1, 17]. More recently, however, interest in the analgesic potential of nAChR ligands has increased with the discovery that epibatidine, a potent nAChR agonist isolated from frog skin by John Daly, is fully efficacious and about 100-fold more potent than morphine in rodent models of acute thermal pain (e.g., hot-plate, tail-flick) [2, 16].

Unfortunately, epibatidine is a toxic compound. The separation between the doses producing antinociception and those producing seizures and death is relatively small in mice, and toxicity is potentiated with repeated dosing [15]. The key to the toxicity of epibatidine may be its high potency across a broad

range of nAChR subtypes, including those present in autonomic ganglia and voluntary musculature [2, 16].

Given the evidence for nAChR diversity that has emerged over the last decade, however, it may be possible to achieve greater separation between desirable and undesirable effects of nAChR activation with improved subtype selectivity [9]. An example of a step in this direction is ABT-594 [10]. This compound has affinity comparable to that of (±)-epibatidine for the high affinity nicotine binding site present in rat brain, but has 3000-fold lower affinity than (±)-epibatidine for the neuromuscular-type nAChR (torpedo electroplax) [7]. Perhaps as a result of this improved selectivity, ABT-594 has better separation between antinociceptive and lethal doses in mice than (±)-epibatidine [6].

As shown in *figure 1*, ABT-594 also produces dose-dependent ameliorative effects in a variety of pain models in rats [3, 4]. ABT-594 increases latencies to respond to acute thermal stimuli in a Hargreaves paw withdrawal apparatus and markedly decreases nocifensive responses to an injection of formalin in the hindpaw. In both cases, these antinociceptive effects are prevented by pretreatment with the nAChR antagonist, mecamylamine, but not by the opioid receptor antagonist, naltrexone. Simi-

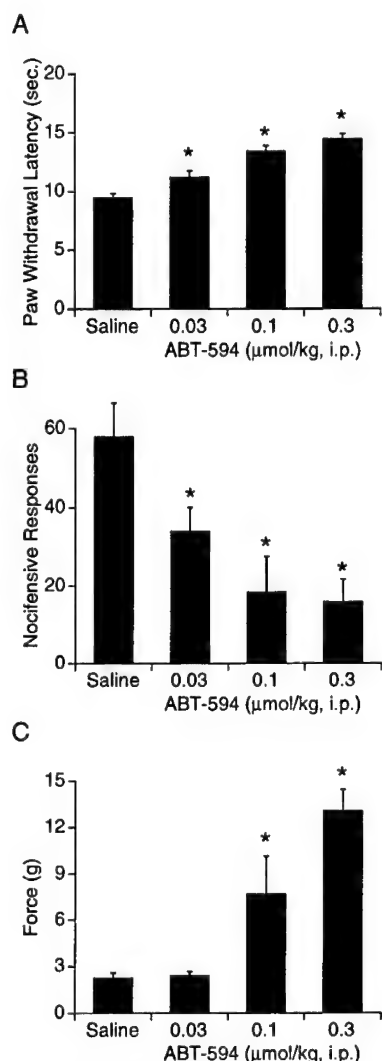


Figure 1. Shown are antinociceptive effects of ABT-594 administered i.p. on measures of acute thermal pain (**A**, paw withdrawal from a focused light source), persistent chemical pain (**B**, formalin injection in hindpaw), and neuropathic pain (**C**, Chung nerve ligation). Data are redrawn from data previously presented in [4]. Shown are means (\pm S.E.M.). *Difference from control, $P < 0.05$.

larly, in the Chung nerve ligation model of neuropathic pain, ABT-594 reverses tactile allodynia in the affected paw. Again, the effect can be prevented by mecamylamine, demonstrating that it is mediated by activation of nAChRs.

The antinociceptive efficacy of ABT-594 is maintained with repeated dosing [3]. In both the paw withdrawal and the neuropathic pain models, the effects of ABT-594 are maintained after a period of

twice daily dosing for 5 days. In contrast, the effects of morphine are reduced under this same dosing schedule, particularly in the neuropathic pain model. Interestingly, effects of ABT-594 on motor performance and body temperature are significantly attenuated with repeated dosing, suggesting that the mechanisms underlying these effects of ABT-594 are distinct from those underlying its antinociceptive actions.

The antinociceptive effects of ABT-594 in the paw withdrawal and formalin models are significantly attenuated by i.c.v. pretreatment with chlorisondamine [3], a compound that produces a long-lasting blockade of central nAChRs. Thus, at least some of the antinociceptive effects of ABT-594 appear to be mediated by interactions with nAChRs in the central nervous system (CNS). One potential central site of action for ABT-594 is the brainstem, a site of origin for multiple descending inhibitory influences on pain transmission [8]. Pharmacological studies suggest that the nucleus raphe magnus (NRM) may be of particular importance in nAChR-mediated antinociception [11, 12]. Consistent with this proposal, systemically-administered ABT-594 increases expression of c-fos in the NRM, and microinjection of ABT-594 into the NRM produces antinociception in the paw withdrawal model [4, 5].

The antinociceptive effects of intra-NRM ABT-594 in the paw withdrawal model are prevented by mecamylamine coadministered into the NRM. Thus, effects of intra-NRM ABT-594 appear to be mediated by nAChRs in the NRM. However, when this dose of mecamylamine is injected into the NRM prior to systemic ABT-594, the antinociceptive effect of ABT-594 is unaltered, suggesting that activation of nAChRs outside of the NRM also plays a role in the antinociceptive effects of systemically-administered ABT-594.

Activation of AChRs can increase release of serotonin in rat brain [13], and intrathecal administration of serotonin antagonists attenuates the antinociceptive effects of systemic (–)-nicotine [14]. These observations, coupled with recent evidence that serotonergic neurons in the NRM express the $\alpha 4$ -containing nAChRs [5], suggest that nAChR-mediated antinociception involves stimulation of serotonergic neurons in the NRM. This hypothesis is supported by the finding that intra-NRM administration of the serotonergic neurotoxin, 5,7-DHT, significantly attenuates the antinociceptive effect of systemically-administered ABT-594 in the paw withdrawal model, albeit incompletely (figure 2). This lesion results in a loss of serotonin-containing neurons in the NRM, as measured by tryptophan hydroxylase immunostaining, but not in the dorsal raphe, so the effect appears to involve the serotonergic

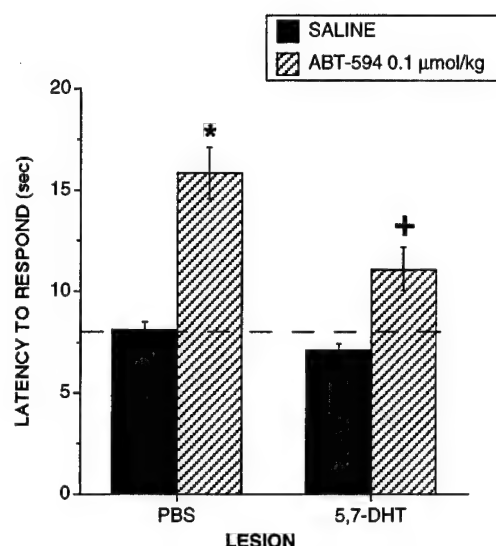


Figure 2. The antinociceptive effects of systemic ABT-594 (0.1 μmol/kg, i.p.) on paw withdrawal latencies were significantly attenuated but not abolished by an NRM lesion 15–22 days prior. For lesion surgery, phosphate buffered saline (PBS; 0.3 μL) or 5,7-DHT (3.75 nmol) was injected into the NRM (coordinates from interaural zero: –2.5 mm A-P, 0.0 mm lateral, and –0.5 mm D-V). Shown are mean latencies to respond (\pm S.E.M.) to an acute thermal stimulus assessed using a commercially available paw thermal stimulator (Dept. of Anesthesiology, Univ. California, San Diego, La Jolla, CA, USA) as previously described [4]. Latencies were measured 15, 30, and 45 min after systemic injections and averaged for presentation. $n = 7$ –8/group. *Difference from PBS/saline ($P < 0.05$); +Difference ($P < 0.05$) from both PBS/ABT-594 and PBS/saline rats.

neurons in the NRM specifically. Interestingly, the lesion does not alter all effects of ABT-594, since no lesion effects on locomotor activity effects of systemic ABT-594 are found.

Whereas a serotonin-specific lesion of the NRM attenuates the antinociceptive effect of systemic ABT-594, reversible inactivation of the NRM with lidocaine does not. The antinociceptive effects of systemic ABT-594 are not prevented by inactivation of the NRM with lidocaine (figure 3A). In contrast, lidocaine-inactivation of the NRM prevents the antinociceptive effects of systemic (–)-nicotine (figure 3B). These results may suggest that the antinociceptive effects of ABT-594 are less dependent on the NRM than are the antinociceptive effects of (–)-nicotine. The distinct nature of the disruption of NRM function by injections of lidocaine and 5,7-DHT may account for the difference in the effects of these two manipulations on ABT-594-induced antinociception. Reversible inactivation of the NRM with lidocaine

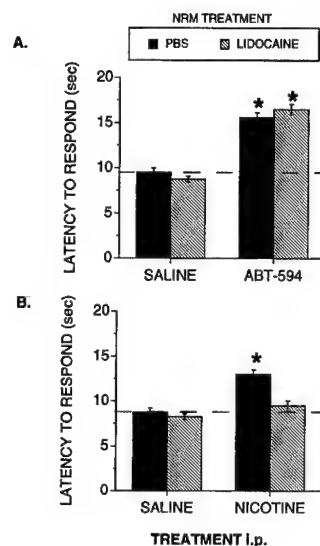


Figure 3. The antinociceptive effects of systemic ABT-594 (0.1 μmol/kg, i.p.) on paw withdrawal latencies were not blocked by an injection of lidocaine (74 nmol in 0.3 μL) into the NRM 5 min prior to ABT-594 (A). However, the antinociceptive effects of systemic (–)-nicotine (1.9 μmol/kg, i.p.) were blocked by intra-NRM lidocaine. Shown are mean latencies to respond (\pm S.E.M.), as described in legend to figure 2. $n = 7$ –8/group. *Difference from PBS control, $P < 0.05$.

may not prevent the release of serotonin in the spinal cord since terminals in the spinal cord are still intact and serotonin could be released by activation of presynaptic nAChRs. In contrast, this potential mechanism would not be available in 5,7-DHT-treated rats with permanent destruction of the NRM-spinal serotonergic pathway.

Overall, these findings suggest that ABT-594 can produce antinociception in an acute thermal pain model by activating nAChRs in the NRM and that serotonergic neurons in the NRM play a role in ABT-594-induced antinociception. However, activation of the NRM is not necessary for the effects of systemically-administered ABT-594, implicating the involvement of other mechanisms. Differences in the effects of NRM lidocaine on antinociception produced by systemic ABT-594 and nicotine may be a reflection of more broadly distributed mechanisms of antinociception for ABT-594 than for (–)-nicotine.

Thus, ABT-594 may be acting at several sites. Effects at other brainstem nuclei, such as the locus coeruleus, are under exploration. In addition, there is the possibility that the compound may act directly on primary afferents. In vitro studies have demonstrated that ABT-594 can attenuate capsaicin-induced release of CGRP from spinal cord slices [7] or from

buccal mucosa (C. Flores, personal communication). Reduction of the release of this 'nociceptive' neuropeptide in both the periphery and the spinal cord suggests that antinociceptive effects of ABT-594 could be mediated by direct actions on either end of nociceptive afferents. This view is supported by the demonstration that the responses of wide dynamic range neurons in the spinal dorsal horn to noxious stimulation can be attenuated by direct administration of ABT-594 into either the spinal cord or the peripheral site of noxious stimulation [4]. Moreover, the effects of ABT-594 appear to be selective for nociceptive afferents since the responses of these same neurons to innocuous stimuli are not affected by ABT-594.

ABT-594 has a broad spectrum of antinociceptive activity in rodent models of pain. Moreover, there are multiple potential mechanisms implicated in the preclinical studies conducted thusfar. One interesting possibility is that distinct mechanisms underlie effects in different pain models and that actions at different nAChR subtypes underlie these diverse effects. This added level of complexity makes the elucidation of mechanisms a more challenging, but also a potentially more rewarding, enterprise.

References

- [1] Aceto M.D., Awaya H., Martin B.R., May E.L., Antinociceptive action of nicotine and its methiodide derivatives in mice and rats, *Br. J. Pharmacol.* 79 (1983) 869–876.
- [2] Badio B., Daly J.W., Epibatidine, a potent analgetic and nicotinic agonist, *Mol. Pharmacol.* 45 (1994) 563–569.
- [3] Bannon A.W., Decker M.W., Curzon P., Buckley M.J., Kim D.J.B., Radek R.J., Lynch J.K., Wasicak J.T., Arnold W.H., Holladay M.W., Arneric S.P., ABT-594 [5-((2R)-azetidinylmethoxy)-2-chloropyridine]: a novel, orally effective antinociceptive agent acting via neuronal nicotinic acetylcholine receptors. II. In vivo characterization, *J. Pharmacol. Exp. Ther.* 285 (1998), 787–794.
- [4] Bannon A.W., Decker M.W., Holladay M.W., Curzon P., Donnelly-Roberts D., Puttfarcken P.S., Bitner R.S., Diaz A., Dickenson A.H., Porsolt R.D., Williams M., Arneric S.P., Broad-spectrum, non-opioid analgesic activity by selective modulation of neuronal nicotinic acetylcholine receptors, *Science* 279 (1998) 77–81.
- [5] Bitner R.S., Nikkel A.L., Curzon P., Arneric S.P., Bannon A.W., Decker M.W., Role of the nucleus raphe magnus in antinociception produced by ABT-594: Immediate early gene responses possibly linked to neuronal nicotinic acetylcholine receptors on serotonergic neurons, *J. Neurosci.* 18 (1998) 5426–5432.
- [6] Decker M.W., Bannon A.W., Buckley M.J., Kim D.J.B., Holladay M.W., Ryther K.B., Lin N.-H., Wasicak J.T., Williams M., Arneric S.P., Antinociceptive effects of the novel neuronal nicotinic acetylcholine receptor agonist, ABT-594, in mice, *Eur. J. Pharmacol.* 346 (1998) 23–33.
- [7] Donnelly-Roberts D.L., Puttfarcken P.S., Kuntzweiler T.A., Briggs C.A., Anderson D.J., Campbell J.E., Manelli A., Piattoni-Kaplan M., McKenna D.G., Wasicak J.T., Holladay M.W., Williams M., Arneric S.P., ABT-594((R)-5-(2-Azetidinylmethoxy)-2-Chloropyridine): I. In vitro profile of a novel analgesic neuronal acetylcholine (nAChR) ligand, *J. Pharmacol. Exp. Ther.* 285 (1998) 777–786.
- [8] Fields H.L., Heinricher M.M., Mason P., Neurotransmitters in nociceptive modulatory circuits, *Annu. Rev. Neuroscience* 14 (1991) 219–245.
- [9] Holladay M.W., Dart M.J., Lynch J.K., Neuronal nicotinic acetylcholine receptors as targets for drug discovery, *J. Med. Chem.* 40 (1997) 4169–4194.
- [10] Holladay M.W., Wasicak J.T., Lin N.H., He Y., Ryther K.B., Bannon A.W., Buckley M.J., Kim D.J.B., Decker M.W., Anderson D.J., Campbell J.E., Kuntzweiler T.A., Donnelly-Roberts D.L., Piattoni-Kaplan M., Briggs C.A., Williams M., Arneric S.P., Identification and initial structure-activity relationships of (R)-5-(2-azetidinylmethoxy)-2-chloropyridine (ABT-594), a potent, orally active non-opiate analgesic agent acting via neuronal nicotinic acetylcholine receptors, *J. Med. Chem.* 41 (1998) 407–412.
- [11] Iwamoto E.T., Antinociception after nicotine administration into the mesopontine tegmentum of rats: Evidence for muscarinic actions, *J. Pharmacol. Exp. Ther.* 251 (1989) 412–421.
- [12] Iwamoto E.T., Characterization of the antinociception induced by nicotine in the pedunculopontine tegmental nucleus and the nucleus raphe magnus, *J. Pharmacol. Exp. Ther.* 257 (1990) 120–133.
- [13] Ribeiro E.B., Bettiker R.L., Bogdanov M., Wurtman R.J., Effects of systemic nicotine on serotonin release in rat brain, *Brain Res.* 621 (1993) 311–318.
- [14] Rogers D.T., Iwamoto E.T., Multiple spinal mediators in parenteral nicotine-induced antinociception, *J. Pharmacol. Exp. Ther.* 267 (1993) 341–349.
- [15] Sullivan J.P., Briggs C.A., Donnelly-Roberts D., Brioni J.D., Radek R.J., McKenna D.G., Campbell J.E., Arneric S.P., Decker M.W., Bannon A.W., (±)-Epibatidine can differentially evoke responses mediated by putative subtypes of nicotinic acetylcholine receptors (nAChRs), *Med. Chem. Res.* 4 (1994) 502–516.
- [16] Sullivan J.P., Decker M.W., Brioni J.D., Donnelly-Roberts D., Anderson D.J., Bannon A.W., Kang C., Adams P., Piattoni-Kaplan M., Buckley M.J., Gopalakrishnan M., Williams M., Arneric S.P., (±)-Epibatidine elicits a diversity of in vitro and in vivo effects mediated by nicotinic acetylcholine receptors, *J. Pharmacol. Exp. Ther.* 271 (1994) 624–631.
- [17] Tripathi H.L., Martin B.R., Aceto M.D., Nicotine-induced antinociception in rats and mice: correlation with nicotine brain levels, *J. Pharmacol. Exp. Ther.* 221 (1982) 91–96.

Neural substrate of nicotine addiction as defined by functional brain maps of gene expression

Emilio Merlo Pich, Cristian Chiamulera, Michela Tessari

Glaxo Wellcome SpA, Pharmacology Directorate, via Fleming 4, 37100 Verona, Italy

Abstract — The distributed neural networks involved in the intravenous self-administration of nicotine and cocaine, and in a model of relapse of nicotine-taking after abstinence, were compared in Wistar rats. Post-mortem brain maps of c-fos-related antigens expression showed specific activation in prefrontal cortex, anterior cingulate and nucleus accumbens for both drugs, but of the anterior cingulate cortex only during relapse, suggesting that a subset of the neural network involved in drug self-administration is activated during relapse. (©Elsevier, Paris)

Résumé — Activation différentielle des régions cérébrales pendant l'auto-injection de la nicotine chez le rat. La mobilisation de différentes structures cérébrales cibles du système dopaminergique mésocorticolimbique chez le rat qui s'injecte nicotine ou cocaïne, ainsi qu'un modèle de rechute de la prise de la nicotine, ont été étudiés avec l'analyse du changement de distribution de l'expression de la famille des facteurs de transcription c-fos. La cortex cingulate antérieure, et non l'accumbens, sera la seule région activée dans les trois modèles. (©Elsevier, Paris)

nicotine / cocaine / drug self-administration / c-fos / FRAs / dopamine / rat

1. Introduction

Nicotine is critical in the maintenance of tobacco smoking. Nicotine, like cocaine, activates the mesocorticolimbic dopamine (DA) system [1, 4]. Experiments with animals that voluntarily press a lever to receive cocaine infusions strongly indicate that the mesocorticolimbic DA system is also a key neuroanatomical substrate for drug-seeking behavior itself. Recent data indicate that also nicotine is self-administered in rats and that the mesocorticolimbic DA system is likely to be involved in nicotine self-administration [2, 10]. However, it is not clear what the involvement of the mesocorticolimbic DA system in the relapse of nicotine self-administration after a period of abstinence is.

A powerful approach to the identification of the neural network implicated in the control of a given behavior is the extensive mapping of brain structures for immediate early gene expression, e.g., c-fos [9] and other members of the c-fos family, the Fos-related antigens [11]. In particular, acute injections of cocaine and nicotine are known to produce transient expression of the c-fos proteins in the nucleus accumbens and striatum, two of the regions of the terminal fields of the mesocorticolimbic DA system.

The goals of our investigations, briefly reviewed in this article, were to produce post-mortem brain maps measuring the expression of c-fos related transcription factors in the terminal fields of the mesocorticolimbic DA system during nicotine self-administration, and to compare them to the maps

produced by: 1) cocaine self-administration; and 2) reinstatement of nicotine self-administration after a period of 'abstinence'.

2. Materials and methods

Wistar rats were individually housed in a temperature-controlled environment on a 12-h light-12-h dark cycle with the light switched on at 6:00 a.m. Food and water was freely available until the beginning of the experiments.

2.1. Nicotine and cocaine self-administration

Behavioral testing was conducted in 16 operant chambers. Each chamber was equipped with two levers. Right lever presses ('active lever presses') produced reinforcement delivery (food pellet or i.v. infusion). Data acquisition and schedule parameters were controlled by a Med-PC software. Self-administration techniques were performed as described by Corrigan et al. [2] with minor modifications. Rats ($n = 4-13$) were first trained to lever press for food as a reinforcer, and then, after surgery for catheter implantation, they were re-trained for nicotine (0.03 µg/kg/infusion), cocaine (0.25 mg/kg/infusion), or saline intravenous self-administration with an FR3 schedule, 1 h/day. One group of rats was not trained for lever press, but only placed in the cage for 1 h. About 2 weeks later, rats were killed 60–90 min after the last self-administration session. Their performances, measured in lever press/h, were: nicotine, 53 ± 10 ; cocaine 49 ± 9 , saline 12 ± 5 ; untrained 6 ± 3 . For more details, see Merle Pich et al. [5].

2.2. Model of relapse of nicotine self-administration after abstinence

To assess the relevance of external cue in relapse of self-administration for nicotine, a model based on classical conditioning was established in rats. A group of rats was trained using an auditory stimulus paired with nicotine infusion using the same self-administration paradigm described above, and operated by the same lever controlling the nicotine injector for self-administration. After stabilization of responding (average: 87.3 ± 20.1 lever presses/h), both nicotine and auditory stimuli were removed for 7 days. During this period of 'abstinence' the frequency of lever press in most rats was significantly reduced (18.9 ± 4.3 , $P < 0.01$), indicating extinction. When a group of rats was re-exposed to the auditory stimulus previously paired with nicotine infusions, the frequency of lever press in absence of nicotine recovered towards pre-'abstinence' values (38.2 ± 8.4 , $P < 0.01$). Rats were killed 90 min after the last session.

2.3. Immunohistochemistry for Fos-like immunoreactivity and image analysis

Ninety min after the end of the last self-administration session, rats were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde. The time chosen for perfusion approximately coincides with the peak levels of Fos antigen after stimuli [8]. C-fos was recognized using a polyclonal sheep antibody (#OA-11-823 Cambridge Research Biochemicals), whereas FRAs were recognized using a polyclonal antibody (gift from Dr. Iadarola). The terminal fields of the mesocorticolimbic DA system were identified using antibodies against tyrosine Hydroxylase (TH, Chemicon), and double-labeling immunocytochemistry was performed using TH and Fos or FRAs antibodies. Computer-assisted morphometrical techniques were used to count the number of Fos-like immunoreactive profiles (IL) in specific anatomical regions in at least three adjacent sections/rat [5, 7] (see figure 1A–C). The anti-FRAs antibody was also used for Western blot analysis performed on tissue extract from dissected brain regions, as previously described [5].

3. Results

In summary, exposure to nicotine self-administration produced an increase of Fos-LI in 43 brain regions when compared to 'sham' untrained control, and in 33 brain regions when compared with rats trained previously for food but then exposed to saline. Among these regions, the anterior cingulate, the infralimbic cortex (see figure 1F), the shell of nucleus accumbens, all regions of the terminal field of the mesocorticolimbic DA system were found significantly activated (figure 2, top) (for more details, see Pagliusi et al. [7]). Interestingly, rats trained for food self-administration and then exposed to saline instead to nicotine showed significant activation when compared with the 'sham' untrained control (figure 2).

Exposure to cocaine self administration did not increase the Fos-LI expression when compared to the saline group (figure 2), a result consistent with previous reports of downregulation of Fos response to repeated injection of cocaine [3]. However, both nicotine and cocaine increased the levels of FRAs-IL in anterior cingulate cortex, infralimbic cortex, shell and core of the

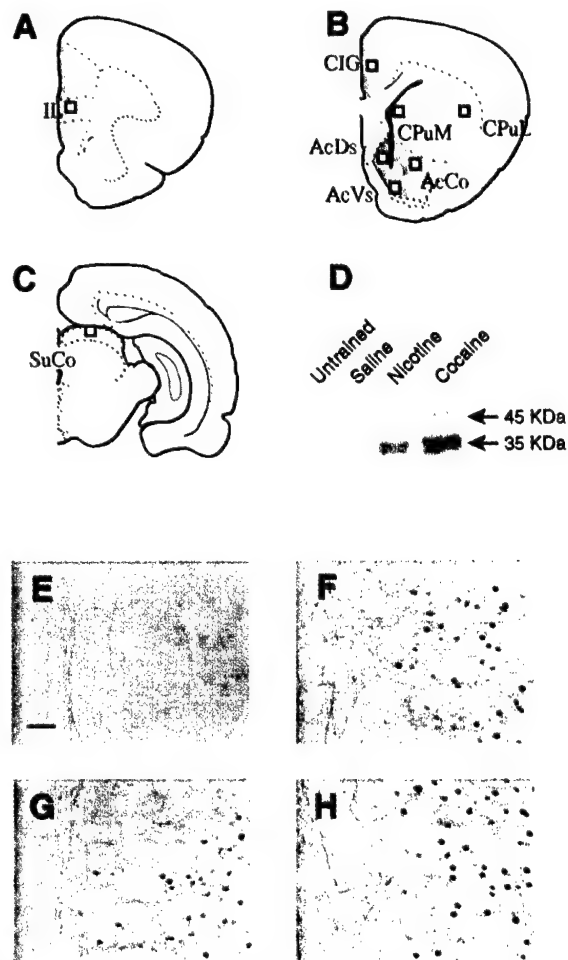


Figure 1. A–C. Cartography of rat brain structures sampled for computer-assisted image analysis. Colored areas indicate the terminal fields of dopamine mesocorticolimbic system, i.e., the prefrontal cortex (blue: IL, infralimbic cortex; CIG, cingulate cortex), the nucleus accumbens (green: AcDs, dorsal shell; AcVs, ventral shell; AcCo, core), the caudate-putamen (yellow: CPuM, medial; CPuL, dorsal), and a region devoid of dopamine terminals but rich in nicotinic receptors, the superior colliculus (orange: external layer, SuCo). D. Western blot using anti-FRAs antibody on extracts from prefrontal cortex (blue). Data from Merle Pich et al. [5]. E, F. Examples of immunocytochemistry for Fos-LI in the infralimbic cortex of 'sham' control rat (E); nicotine self-administration rat (F). G. Saline rat. H. Cocaine self-administration rat. Calibration bar 50 μm.

nucleus accumbens, medial caudate, but not amygdala, all terminal fields of the mesocorticolimbic DA system, while only cocaine activated the dorsolateral striatum, the terminal field of the nigrostriatal DA system (figure 2, bottom). As shown with Fos-LI, some increase of FRAs-LI was observed in rats trained for food but exposed to saline instead of nicotine or cocaine, suggesting that motivational learning of an operant task can activate the same structures strongly activated by nicotine or cocaine. Gel-shift assays and Western blots on dissected brain structures confirmed these results (figure 1D) (for more details, see Merle Pich et al. [5]).

When mapping of Fos-IL expression was performed in brains of rats exposed to the acoustic cue previously paired with nicotine infusion after a period of 'abstinence', an increase of Fos-IL profiles

was found neither in the accumbens nor in the infralimbic cortex, but only in anterior cingulate cortex (Chiamulera, in preparation).

4. Discussion

Functional mappings by immediate early genes, in particular Fos and FRAs, provide the identification of neurons transcriptionally active during nicotine self-administration. Newly translated Fos and FRAs heterodimerize with members of the Jun family to form the activating protein-1 (AP-1) complexes, important transcriptional regulators in neurons [6]. Interestingly some FRAs, i.e., the 35 kDa component, does not behave as immediate early

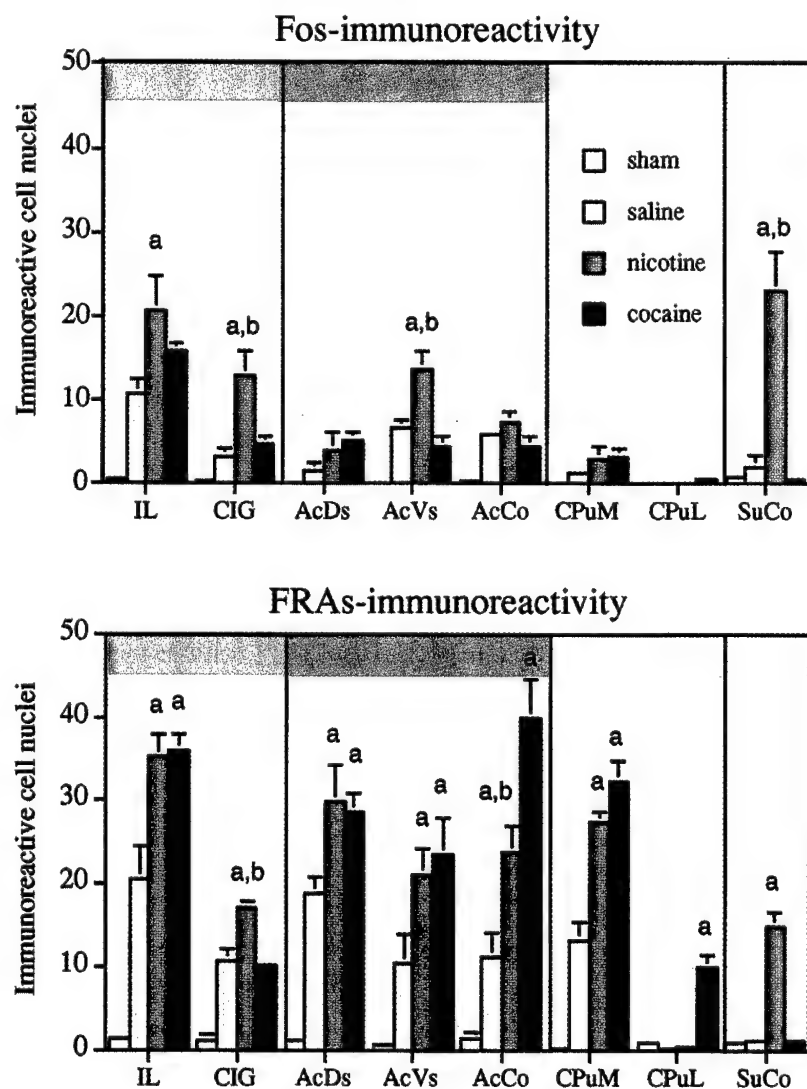


Figure 2. Counts of nuclei of neurons positively stained for Fos-LI (upper panel) and FRAs-LI (lower panel) in the brain regions described in figure 1. The colored strip on the top of the histograms indicates the brain areas colored in figure 1. Analysis of variance showed significant difference versus saline ('a', $P < 0.01$), or versus cocaine self-administration ('b', $P < 0.01$). Saline, nicotine self-administration and cocaine self-administration groups were significantly different in prefrontal cortex and nucleus accumbens from 'sham' control group ($P < 0.01$, not shown). Part of these data are from Merle Pich et al. [5].

genes, but its product, once induced in neurons, may last for several days [3]. In our studies two antibodies, one that was mainly recognizing Fos (55 kDa) [7], the other recognizing FRAs, and in particular the 35 kDa FRA band [3, 11], were used to identify to substrates of the reinforcing effects of nicotine and cocaine. The two drugs produced largely overlapping maps of 35 kDa FRAs on the terminal fields of the mesocorticolimbic DA system (see also *figure 1D*), indicating a prolonged stimulation of these neurons, and confirming the central role of such system in the motivational aspect of drug-taking. Since repeated cocaine exposure produces a downregulation of Fos response (*figure 2*, top), no comparison was possible between short-term maps of Fos produced by nicotine self-administration versus cocaine self-administration.

However, Fos mapping was used to compare the effects of an external cue paired with nicotine infusion after a period of 'abstinence' in a model of nicotine-taking relapse. Comparisons between the brains of rats exposed or not exposed to the conditioned stimulus alone after 1 week of 'abstinence' revealed that, among the various terminal fields of the mesocorticolimbic DA system, only the anterior cingulate cortex was activated, whereas the nucleus accumbens was not.

In conclusion, the Fos maps of self-administration behavior produced by the presentation of a conditioned cue previously associated with the reinforcing effect of nicotine were more restrictive than the Fos maps of nicotine self-administration, supporting the anterior cingulate cortex involvement in association between the external cue and the reinforcing effect of nicotine.

Acknowledgments

The authors of this review acknowledge the contribution given by S. Pagliusi, D. Talabot-Ayer, R. Hoof van Huijsdui-

jen, and C. Hebert. All work was supported by Galxo Wellcome SpA, Verona, and GBRI Glaxo Wellcome R&D, Geneva.

References

- [1] Calabresi P., Lacey M.G., North R.A., Nicotinic excitation of rat ventral tegmental area neurones in vivo studied by intracellular recording, *Br. J. Pharmacol.* 98 (1989) 135-140.
- [2] Corrigal W.A., Coen K.M., Adamson K.L., Self-administered nicotine activates the mesolimbic dopamine system through the ventral tegmental area, *Brain Res.* 653 (1994) 278-284.
- [3] Hope B.T., Nye H.E., Kelz M.B., Self D.W., Iadarola M.J., Nakabepu Y., Duman R.S., Nestler E.J., Induction of a long lasting AP-1 complex composed of altered Fos-like proteins of brain by chronic cocaine and other chronic treatments, *Neurons* 13 (1994) 1235-1244.
- [4] Imperato A., Mulis A., DiChiara G., Nicotine preferentially stimulates dopamine release in the limbic system of freely moving rats, *Eur. J. Pharmacol.* 132 (1986) 337-338.
- [5] Merlo Pich E., Pagliusi S., Tessari M., Talabot-Ayer D., Hoffit van Huijsduijn, Chiamulera C., Common neural substrates for the addictive properties of nicotine and cocaine, *Science* 275 (1997) 83-86.
- [6] Morgan I., Curran T., Stimulus transcription coupling in neurons: role of cellular immediate early genes, *TINS* 12 (1989) 459-462.
- [7] Pagliusi S.R., Tessari M., DeVevey S., Chiamulera C., Merlo Pich E., The reinforcing properties of nicotine are associated with a specific patterning of c-fos expression in the rat brain, *Eur. J. Neurosci.* 8 (1996) 2247-2256.
- [8] Ren T., Sagar S.M., Induction of c-fos immunostaining in the rat brain after the systemic administration of nicotine, *Brain Res. Bull.* 29 (1992) 589-597.
- [9] Sagar S.M., Sharp F.R., Curran T., Expression of c-fos-protein in brain: metabolic mapping at the cellular level, *Science* 240 (1988) 1328-1331.
- [10] Tessari M., Valerio E., Chiamulera C., Beardsley P.M., Nicotine reinforcement in rats with histories of cocaine self-administration, *Psychopharmacology* 121 (1995) 282-283.
- [11] Young S.T., Porrino L.J., Iadarola M.J., Cocaine induces striatal c-fos immunoreactive proteins via dopaminergic D1 receptors, *PNAS* 88 (1991) 1291-1295.

Is dopamine important in nicotine dependence?

Mohammed Shoaib

Section of Behavioural Pharmacology, Institute of Psychiatry, DeCrespigny Park, London SE5 8AF, UK

Abstract — Nicotine, like other drugs when abused, can produce a wide array of behaviours, some of which collectively propel 'drug-seeking behaviour'. This review focuses on three stimulus properties of nicotine and examines the role of dopamine in mediating each effect with respect to D1 and D2 receptor subtypes. Dopamine appears to be critical in mediating the reinforcing effects of nicotine, which is in line with other commonly abused psychomotor stimulants. However, evidence derived from studies with local microinjections of nicotine suggests that the origin of nicotine action to produce its other stimulus properties may be via multiple neuroanatomical substrates. The aversive stimulus effects are resistant to dopamine receptor antagonists. The discriminative stimulus effects of nicotine, despite showing some modification with dopaminergic compounds, appear not to be solely mediated via the mesolimbic dopamine system. Taken together, the neurobiology of nicotine dependence remains complex. Nonetheless, such dissociation between stimulus properties may permit the development of more effective therapies in combating tobacco dependence. (©Elsevier, Paris)

Résumé — La dopamine intervient-elle dans la dépendance à la nicotine? Comme d'autres drogues induisant une dépendance, la nicotine peut induire des comportements variés, dont l'ensemble produit un 'comportement de recherche de drogue'. Cette revue concerne principalement trois activités stimulatrices de la nicotine et le rôle éventuel de la dopamine dans ces effets, en relation avec les récepteurs D1 et D2. La dopamine est essentielle pour les effets de renforcement, ce qui est aussi observé avec d'autres drogues produisant une stimulation psychomotrice. Cependant, les effets de microinjections locales de nicotine suggèrent que les actions stimulantes de la nicotine passent par de multiples voies neuroanatomiques. Les stimuli répulsifs résistent aux antagonistes des récepteurs dopaminergiques. Les effets discriminatifs de la nicotine sont peu affectés par les agents dopaminergiques et ne semblent pas faire exclusivement intervenir le système mésolimbique dopaminergique. La neurobiologie de la dépendance à la nicotine est donc complexe. Le fait qu'il soit possible de dissocier les différents effets de la nicotine pourrait permettre de mettre au point de nouvelles thérapies pour lutter contre la dépendance au tabac. (©Elsevier, Paris)

reinforcing stimulus / discriminative stimulus / aversive stimulus / neurobiology / mesolimbic dopamine / rodent

1. Introduction

The neurotransmitter dopamine, by far, has occupied a centre stage as the key neural substrate mediating the dependence-producing effects of most commonly abused substances. With cocaine and amphetamines, dopamine is regarded as the critical substrate; manipulations of dopaminergic systems produce dramatic changes in behaviour. However, the evidence to support the involvement of this catecholamine in other drug classes has generated some concern. In line with the proposal that all abused substances share the ability to elevate dopamine levels in the nucleus accumbens [5], the alkaloid nicotine found ubiquitously in tobacco products, was also observed to elevate dopamine levels [6]. This primary observation has led authors to categorise nicotine alongside other illicit substances [11]. Not surprisingly, nicotine has dependence-producing properties; it produces a diverse spectrum of behavioural effects, some of which contribute significantly towards the development of dependence. However, it has since become apparent that dopamine may not mediate all of these behaviours. This paper reviews and presents evidence that supports the notion that

dopaminergic processes may not be the sole neural substrate in mediating the behavioural effects of nicotine. In addition to the positive reinforcing stimulus effects of nicotine, the other stimulus properties namely the discriminative and aversive properties, which also play an important role in maintaining and regulating drug intake [21], are considered.

2. Positive reinforcing effects

The ability of nicotine to serve as a positive reinforcer is indicative of all drugs with abuse liability. In humans and laboratory animals, intravenous nicotine, under certain conditions can maintain self-administration behaviour (further details reviewed by [18]). Two key neuroanatomical studies conducted in rodents suggest that a strong dopaminergic basis underlie the reinforcing properties of nicotine. Bilateral lesions of the dopamine terminal regions, the nucleus accumbens (NAC) markedly attenuated the self-administration of nicotine in rats. The neurotoxin 6-hydroxy-dopamine depleted dopamine by approximately 90% in the nucleus accumbens, while only 25% dopamine was lost in the caudate nucleus. Mo-

reover, the same lesions produced modest changes upon responding for food [2]. These results supported earlier findings from the same authors who had examined dopamine receptor antagonists on nicotine self-administration. The dopamine selective D1 SCH23390 and D2 receptor antagonist spiperone both attenuated responding maintained by intravenous nicotine. However, the administration of these antagonists also reduced both operant responding for food and locomotor activity [1]. Despite these non-specific effects of dopamine antagonists, it was argued that the motor impairment was not the cause of decreases in nicotine self-administration since responding was attenuated in the latter half of the sessions. A study conducted in cigarette smokers confirmed the involvement of dopamine receptors in the rewarding effects of nicotine. Compared to placebo, the administration of the dopamine antagonist haloperidol increased blood levels of nicotine, an effect thought to reflect a compensatory increase in smoking in order to obtain the rewarding effect of nicotine [4].

3. Aversive stimulus properties

As discussed above, the positive reinforcing effects of nicotine represent the key element that drives drug-taking behaviour, however, paradoxically nicotine can also serve as an aversive stimulus. These aversive effects have been implicated in the regulation of nicotine intake, perhaps by setting an upper limit to the amounts consumed [9, 15]. Conditioned taste aversions have been used to demonstrate the aversive stimulus properties of a drug, in which rats will readily learn to avoid a distinctively flavoured solution when its consumption was previously paired with administration of nicotine [7, 10].

In the context of dopamine, studies have been limited to those with dopamine receptor antagonists. Indirect evidence has also come from studies inducing taste aversions by microinjecting nicotine locally into dopaminergic nerve terminal regions. *Figure 1* illustrates results from such a taste aversion conditioning experiment in which nicotine was injected into a variety of brain regions [17]. Following two conditioning trials, CTAs were observed in two groups that had received injections into the interpeduncular nucleus or the nucleus accumbens. However, only intraaccumbens administered nicotine produced CTA consistently; an effect that was blocked by mecamylamine, indicating a nicotinic receptor mediated phenomenon. This result was initially perceived as intriguing given the role of the nucleus accumbens in mediating the positive reinforcing effects. Nonetheless, several investigators have shown that the nucleus accumbens can also me-

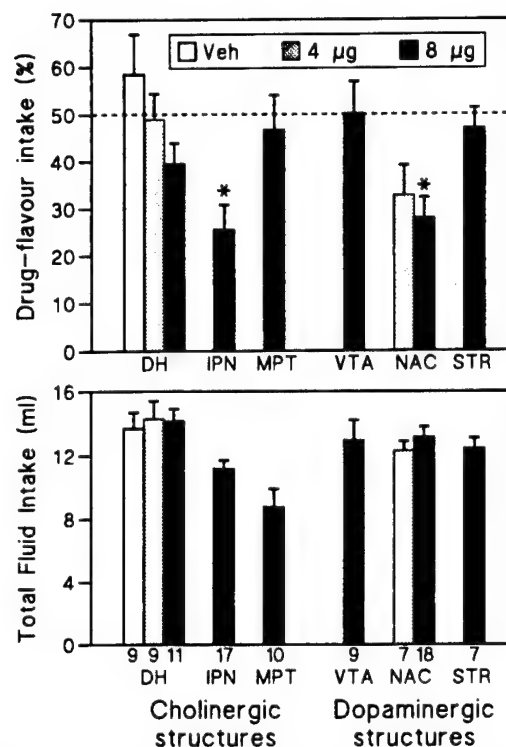


Figure 1. Taste aversion conditioning with nicotine injected into dorsal hippocampus (DH), interpeduncular nucleus (IPN), mesopontine tegmentum (MPT), ventral tegmental area (VTA), nucleus accumbens (NAC) or striatum (STR). The top section shows the percentage of total fluid intake that was in form of drug-paired flavoured solution in two-stimulus tests where both nicotine- and saline-paired flavoured solutions were presented simultaneously; asterisks denote significant CTA ($P < 0.001$) consumption less than 50%. The lower section shows the total fluid intake; the number of rats for each region is shown on the horizontal axis. Reproduced with permission from Shoaib and Stolerman [17].

diates aversive effects of drugs [16] or of drug withdrawal states [8, 20]. However, the CTAs induced by intra-accumbens administration of nicotine do not provide direct evidence that it is mediated by dopamine.

In an early study, doses of the dopamine D2 receptor antagonist pimozide that blocked apomorphine-induced CTAs were ineffective against nicotine-induced CTA [12]. Furthermore, pretreatment with the D1 receptor antagonist SCH23390 failed to block nicotine-induced CTAs. *Figure 2* shows the effects of pretreatment with various doses of SCH23390 (0.01–0.09 mg/kg SC) administered 30 min prior to nicotine injection (0.4 mg/kg SC). In all four groups, rats showed significant avoidance of the flavoured solutions previously paired with ni-

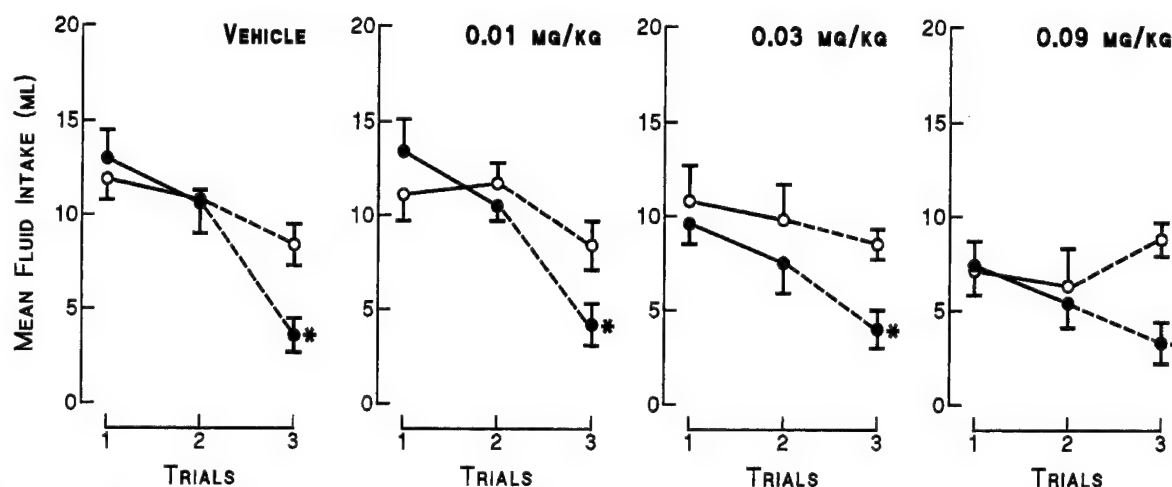


Figure 2. Effect of SCH23390 pretreatment on nicotine-induced conditioned taste aversions ($n = 8$). Trials 1 and 2 were conditioning sessions and trial 3 was two-stimulus test with simultaneous presentation of both flavoured solutions. Vertical bars represent the mean \pm S.E.M. The lines show intakes of flavoured solutions associated with nicotine (●) or saline (○) for each group of rats. The far left figure shows a conditioned taste aversion induced by nicotine (0.4 mg/kg SC). The other figures show taste aversions induced by the same dose of nicotine that was not blocked by various doses of SCH23390 (0.01–0.09 mg/kg SC) administered 30 min prior to presentation of flavoured solutions on conditioning trials 1 and 2.

cotine administration (figure 2). A one-way ANOVA confirmed the apparent absence of effect by SCH23390 pretreatment [$F(3,28) = 0.19$, *n.s.*]. The doses of SCH23390 were behaviourally active since SCH23390 significantly reduced the amounts of fluid consumed during the conditioning trials [$F(3,28) = 21.2$, $P < 0.0001$].

4. Discriminative stimulus effects

The observation that humans and laboratory animals are capable of discriminating nicotine from saline has been thought to be an important attribute of a drug that supports drug-seeking behaviour. In studies exploring the neural basis of nicotine discrimination, Rosecrans and Meltzer [14] demonstrated dosedependent generalisation to intracerebral microinjections of nicotine in rats trained to discriminate systematically administered nicotine. Both the dorsal hippocampus and the reticular formation were implicated as neural substrates of the discriminative properties of nicotine. More recently, Shoaib and Stolerman [19] confirmed the involvement of the dorsal hippocampus as a mediator of the discriminative stimulus function. Infusion of similar doses of nicotine into the mesolimbic dopamine terminal failed to produce nicotine-like effects. Figure 3 illustrates the degree of substitution observed with intrahippocampal and intra-nucleus accumbens injections.

The lack of effect associated with local injection of nicotine into the mesolimbic dopamine system appears inconsistent with findings using dopamine receptor agonists and antagonists. Both D1 and D2 receptor antagonists have been shown to attenuate the discriminative stimulus effect of nicotine [13], however Corrigall et al. [3] have suggested that the effects of dopamine receptor antagonist effects are nonspecific. Moreover, the same study showed an absence of substitution of nicotine to GBR 12909, a dopamine reuptake inhibitor [3]. With dopamine receptor agonists, partial substitution was observed with SKF 38393, a D1 agonist, while no significant effect was observed with quinpirole, a D2/D3 agonist [13]. To date, no studies have examined the influence of neurotoxin lesions to resolve the involvement of dopamine in the cueing effects of nicotine.

5. Discussion

From the evidence presented in this short review it is clear that like other abused drugs, nicotine also relies on dopamine to mediate its reinforcing effects via the mesolimbic pathway originating from the ventral tegmental area and projecting to the nucleus accumbens. However, this projection may not be critical in the transduction of the other stimulus properties of nicotine. The aversive stimulus despite showing some involvement with the nucleus accumbens may rely upon non-dopaminergic processes.

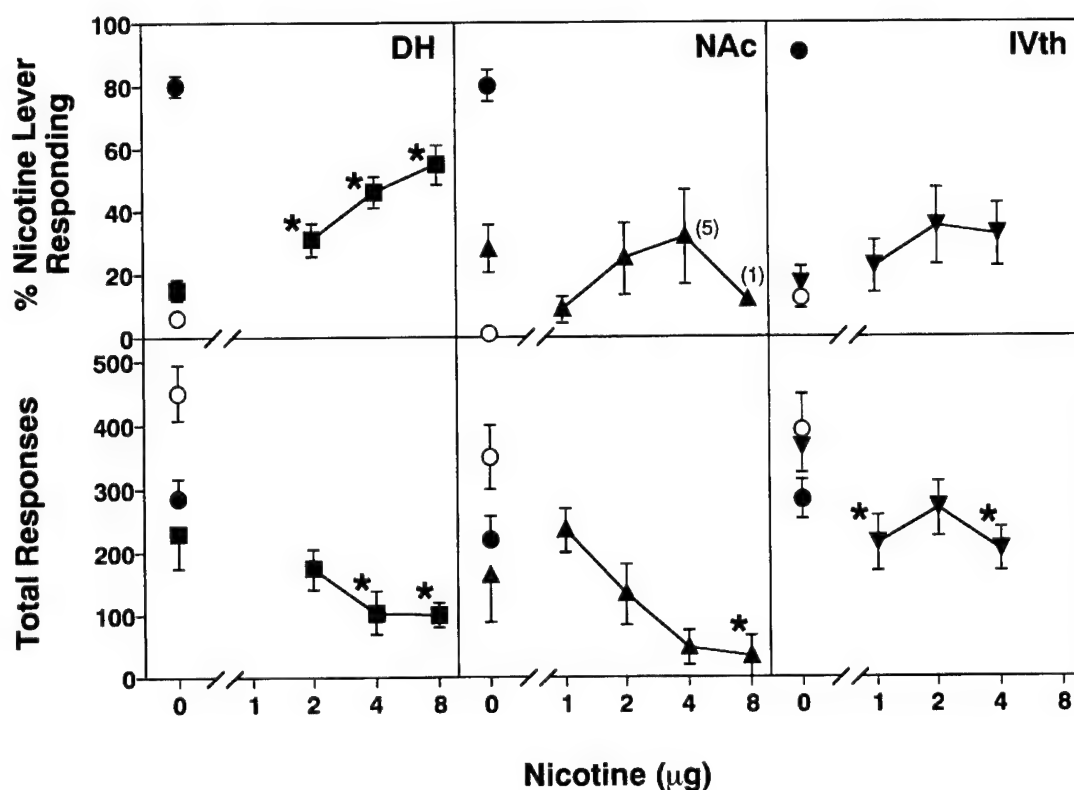


Figure 3. Effect of administering nicotine into the dorsal hippocampus (DH, ■), nucleus accumbens (NAc, ▲), and the fourth ventricle (IVth, ▼) on percent responding on nicotine-appropriate lever and on total number of responses for groups of 9–10 rats trained to discriminate nicotine (●) from saline (○). Points above 0 on the abscissae show results for the subcutaneous injections of saline and nicotine (0.2 mg/kg) and for intracerebral injections of vehicle. Each point represents the mean \pm S.E.M. of observations made in 5-min extinction tests. Numbers in parentheses represent the mean number of animals in cases where suppression of responding precluded collection of data for discriminative effects for some rats. Asterisks denote significant increase in comparison with microinjection of vehicle or significant decreases in total responses as determined with Dunnett's *t*-test ($P < 0.05$). Reproduced with permission from Shoaib and Stolerman [17].

The neural substrate for this stimulus has yet to be fully elucidated. What is more puzzling is the observation that dopamine receptor agonists and antagonists can produce or modify the discriminative stimulus effects of nicotine, while no generalisation is observed following the local application of nicotine into dopaminergic nerve terminals. It would appear that dopamine may not be important in the aversive and cueing properties of nicotine. Such dissociation between stimulus properties presents a potential opportunity to harness in treating nicotine-based addictions.

Acknowledgments

The research trusts (Medical Research Council, UK and the Wellcome Trust, UK) are thanked for their generous support of the research conducted by the author.

References

- [1] Corrigall W.A., Coen K.M., Selective dopamine antagonists reduce nicotine self-administration, *Psychopharmacology* 104 (1991) 171–176.
- [2] Corrigall W.A., Franklin K.B.J., Coen K.M., Clarke P.B.S., The mesolimbic dopaminergic system is implicated in the reinforcing effects of nicotine in rats, *Psychopharmacology* 107 (1992) 285–292.
- [3] Corrigall W.A., Coen K.M., Dopamine mechanisms play at best a small role in the nicotine discriminative stimulus, *Pharmacol. Biochem. Behav.* 3 (1994) 817–820.
- [4] Dawe S., Gerada C., Russell M.A.H., Gray J.A., Nicotine intake in smokers increases following a single dose of haloperidol, *Psychopharmacology (Ber.)* 117 (1995) 110–115.
- [5] Di Chiara G., Imperato A., Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats, *Proc. Natl. Acad. Sci. USA* 85 (1988) 5274–5278.

- [6] Imperato A., Mulas A., Di Chiara G., Nicotine preferentially stimulates dopamine release in the limbic system of freely moving rats, *Eur. J. Pharmacol.* 132 (1986) 337-338.
- [7] Iwamoto E.T., Williamson E.C., Nicotine-induced taste aversion: characterization and preexposure effects in rats, *Pharmacol. Biochem. Behav.* 21 (1984) 527-532.
- [8] Koob G.F., Wall T.L., Bloom F.E., Nucleus accumbens as a substrate for the aversive stimulus effects of opiate withdrawal, *Psychopharmacology* 98 (1989) 530-534.
- [9] Kumar R., Stolerman I.P., Experimental and clinical aspects of drug dependence, in: Iversen L.L., Iversen S.D., Snyder S.H. (Eds.), *Handbook of Psychopharmacology Vol. 7*, Plenum, New York, 1977, pp. 321-367.
- [10] Kumar R., Pratt J.A., Stolerman I.P., Characteristics of conditioned taste aversion produced by nicotine in rats, *Br. J. Pharmacol.* 79 (1983) 245-253.
- [11] Pontieri F.E., Tanda G., Orzi F., Di Chiara G., Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs, *Nature* 382 (1996) 255-257.
- [12] Pratt J.A., Stolerman I.P., Pharmacologically specific pretreatment effects on apomorphine-mediated conditioned taste aversions in rats, *Pharmacol. Biochem. Behav.* 20 (1984) 507-511.
- [13] Reavill C., Stolerman I.P., Interaction of nicotine with dopaminergic mechanisms assessed through drug discrimination and rotational behaviour in rats, *J. Psychopharmacol.* 1 (1987) 264-273.
- [14] Rosecrans J.A., Meltzer L.T., Central sites and mechanisms of action of nicotine, *Neurosci. Biobehav. Rev.* 5 (1981) 497-501.
- [15] Russell M.A.H., Tobacco dependence: Is nicotine rewarding or aversive?, in: Krasnegor N.A. (Ed.), *NIDA Research Monograph 23*, Dept. of Health, Education and Welfare, Washington, 1979, pp. 100-122.
- [16] Shippenberg T.S., Bals-Kubik R., Huber A., Herz A., Neuroanatomical substrates mediating the aversive effects of D-1 dopamine receptor antagonists, *Psychopharmacology* 103 (1991) 209-214.
- [17] Shoaib M., Stolerman I.P., Conditioned taste aversions in rats after intracerebral administration of nicotine, *Behav. Pharmacol.* 6 (1995) 375-385.
- [18] Shoaib M., Determinants of nicotine self-administration, *Drug Dev. Res.* 38 (1996) 212-221.
- [19] Shoaib M., Stolerman I.P., Brain sites mediating the discriminative stimulus effects of nicotine in rats, *Behav. Brain Res.* 78 (1996) 183-188.
- [20] Stinus L., LeMoal M., Koob G.F., Nucleus accumbens and amygdala are possible substrates for the aversive stimulus effects of opiate withdrawal, *Neuroscience* 37 (1990) 767-773.
- [21] Stolerman I.P., Drugs of abuse, behavioural principles, methods and terms, *Trends Pharmacol. Sci.* 13 (1992) 170-176.

Brain choline has a typical precursor profile

Konrad Löffelholz

Department of Pharmacology, University of Mainz, Obere Zahlbacher Str. 67, 55101 Mainz, Germany

Abstract — Choline is product and precursor to both acetylcholine and membrane phospholipids, and, in the brain, is ultimately provided by the circulation. The brain is protected from excess choline and choline deprivation by a refined system of homeostatic mechanisms that maintain a level of extracellular choline that, for its role as precursor, meets saturation criteria under normal conditions. The kinetic and activity profiles of choline are typical for a biosynthetic precursor. (©Elsevier, Paris)

Résumé — La choline cérébrale a un profil typique de précurseur. La choline est à la fois le produit et le précurseur de l'acétylcholine et des phospholipides membranaires et, dans le cerveau, est finalement fourni par la circulation. Les profils cinétiques et d'activité de la choline sont typiques d'un précurseur de biosynthèse. (©Elsevier, Paris)

choline / acetylcholine / cholinergic mechanisms / brain

1. Brain choline: a precursor and an agonist?

Choline (Ch) is the biosynthetic precursor to phospholipids, platelet-activating factor and acetylcholine (ACh). As to the role of Ch in regulating cholinergic mechanisms, it has been discussed for almost a century whether endogenous choline not only serves as a precursor to ACh but also is an agonist at muscarinic and nicotinic receptors under in vivo conditions (see [28, 37]). Clear-cut evidence for agonist properties at both receptor types had been obtained even before Otto Loewi in 1921 detected the neuronal release of the 'Vagusstoff'. The question: "Is Ch an in vivo agonist?" is difficult to answer for two reasons: firstly, agonist effects have been observed only in vitro using high concentrations of Ch. Secondly, there is a lack of specific Ch antagonists that allow to discriminate between effects of Ch and ACh.

Muscarinic peripheral responses of heart, intestine and blood pressure have been described for Ch at concentrations between 40 and 8000 μM (see [28]). The frequently observed enhancement of brain ACh levels by Ch administration which is presumably due to a precursor-induced enhancement of the biosynthesis of ACh ([3]; see below) had been explained alternatively by a central muscarinic response due to the agonist properties of Ch [9].

Nicotinic effects of Ch on blood pressure, sympathetic ganglia and skeletal muscle preparations have been studied frequently in the past; effective concentrations were above 100 μM (see [28, 37]). Recently a specific activation of the $\alpha 7$ -subtype of nicotinic receptor by Ch was detected in neuronal cells [1, 31], the EC_{50} being 1.58 mM. Almost the same values have been reported for displacement

studies using L- ^3H -nicotine binding in various brain regions ($K_i = 1.3 \text{ mM}$) and for the Ch-induced release of the adrenal hormone ($\text{EC}_{50} = 1.3 \text{ mM}$; [37]). Alkondon et al. [1] suggested that Ch may act in vivo as an endogenous specific ligand for these nicotinic receptors and thereby may contribute to the glutamatergic initiation of long-term-potential (LTP).

The present study summarizes the evidence that the kinetic profile of brain choline is typical for a nutrient precursor and does not lend support for a role as agonist.

2. Choline levels under resting conditions

The plasma level of free Ch under resting conditions is remarkably constant at around 10 μM in man and mammals. Ch-deficient diet lowers the plasma level to about 5 μM , whereas a Ch-rich diet leads to plasma levels of about 15 μM [16, 42]. Excessive intake of egg or soy lecithin caused a transient increase of maximally up to 40 μM [42]. These figures are remarkably constant at a low level considering the large reservoir of intracellular Ch ($\geq 50 \mu\text{M}$) and of Ch bound to phospholipids in the plasma ($> 1 \text{ mM}$) and in cell membranes. The efficiency of homeostatic mechanisms regulating the concentration of free Ch in the plasma is obvious, but still allows dietary fluctuations ranging from 5 to 15 μM (under extreme conditions up to 40 μM). Such dietary fluctuations of the plasma level are typical for essential nutrients such as Ch.

In the brain, homeostatic mechanisms (see below) protect the tissue even from the above dietary fluctuations of plasma Ch levels [11, 27]. In the phy-

biological range of 5–15 μM the average extracellular concentration of Ch reflected by the CSF choline was kept constant at approximately 4–6 μM (Tuček [35], estimated 3 μM). The threshold plasma level leading to a rise in free brain Ch was almost 100 μM (after i.p. injection of 60 mg/kg). Ch-deficient diet had no effect on the CSF Ch, although the plasma level was reduced to about 5 μM . Only chronic Ch-supplemented diet led to a significant, but small increase in the CSF Ch level (15.3 μM [16]).

The assumption that the extracellular Ch level is basically similar throughout the brain irrespective of a cholinergic innervation is evident from a large number of studies (e.g., [19, 20, 34]).

3. Homeostatic control of brain choline

Ch is transported through the blood-brain barrier by a choline transport system ($K_m = 442 \mu\text{M}$; $V_{\max} = 10 \text{ nmol/g/min}$ [4]) which is unsaturated at physiological plasma levels (10 μM). An increase of brain Ch uptake due to acute dietary elevation of the plasma level does not enhance extracellular Ch because newly taken up Ch is rapidly removed by brain cells [10, 11] preferentially by glial cells [41]. It deserves mention that glial cells in the brain are about 10 times more numerous than neurons. Koshimura et al. [20] injected 10 μmol Ch intracerebroventricularly and found a drastic increase of the rat striatal Ch content from 60 to 500 nmol/g, whereas the extracellular free Ch level as determined by the microdialysis technique showed a 50% increase only. After cellular uptake, surplus Ch is phosphorylated and slowly incorporated into phospholipids [11]. Uptake with subsequent phosphorylation is rapid and efficacious and protects the brain from increases in extracellular and intracellular free Ch levels.

Surplus bound Ch is eliminated from the brain as free Ch by a protracted release into the venous blood resulting in a negative arterio-venous difference [10, 11, 16, 27]. Ch net-release from the brain and net-uptake into the brain are in a dynamic equilibrium. Thus the negative arterio-venous difference is reversed into the positive (net-uptake of choline) when the plasma level rises due, for example, to acute dietary intake; the 'reversal point' was found to be about 15 μM in the rat [10]. Chronic Ch supplementation, which only slightly elevated the plasma level from 10 to 14 μM , drastically shifted the reversal point from 15 to 40 μM [16]. This result demonstrates the remarkable adaptation of the brain Ch homeostasis to chronic Ch load.

Knowledge about the regulation of uptake and release of free Ch on a cellular level is essential for the understanding of the brain homeostatic control of free extracellular Ch inasmuch as there is at least

10 times more intracellular Ch [36] and about 600 times more bound Ch than extracellular Ch. This holds true even more so for the control of the synaptic concentration of free Ch.

4. Cellular uptake and release of choline in the brain

The transmembrane gradient of free Ch (> 10) is dependent on the membrane potential and the high-affinity Ch uptake (HACU; $K_m = 1\text{--}2 \mu\text{M}$) present in cholinergic nerve terminals [36]. Tuček [36] suggested that the transmembrane gradient is due to the positive charge of Ch and the electronegativity on the inside of the plasma membrane. For example, HACU is reduced during K^+ -depolarization, but is markedly enhanced at a time when the ACh store is depleted and the terminal is allowed to repolarize; this enhancement may be due to after-hyperpolarization and/or to mass action [38]. Protein kinases phosphorylating the transporter may regulate the V_{\max} of the HACU [39]. Whatever mechanism controls the transmembrane gradient, there are various important mechanisms of cellular uptake and release of Ch.

In nervous tissue, extracellular Ch is taken up into neurons and glial cells by specific cellular uptake mechanisms. Cholinergic nerve endings possess the hemicholinium (HC-3)-sensitive HACU and a low-affinity uptake. It seems, that essentially the HACU is associated with ACh synthesis under normal conditions [39]. Also glial cells are equipped with some kind of a HACU [29]. In the leech central nervous system, electrophysiological and autoradiographic studies provided good evidence that glial cells more effectively accumulate Ch than neurons; ACh itself was not taken up but was metabolized to Ch which was subsequently removed by glial cells [41].

As mentioned above, surplus bound Ch (e.g., phosphatidylcholine) is eliminated by release of free Ch into the extracellular space and back into the blood via the CSF compartment [16, 27]. There are many conditions, such as hypoxia, elevation of Ca^{2+} , receptor-activation by transmitters and hormones, which enhance the extracellular Ch level by mobilizing free intracellular Ch or bound Ch. Receptor-activation of phospholipases (PLs), such as PLA_2 and PLD, enhance the release of choline from phospholipids. For the first time, a receptor-mediated release of Ch was observed in the isolated chicken heart and in the rat cortex in vivo [5, 23]. Unexpectedly physostigmine, bethanechol and oxotremorine enhanced the release of Ch from rat cortex and brain in vivo in an atropine-sensitive manner [2, 5, 14]; these observations were confirmed using the mi-

cro-dialysis technique in frontal cortex, hippocampus and striatum [34]. The muscarinic increase of the extracellular Ch concentration was apparently due to activation of PLD [13, 25]. Likewise glutamatergic activation of PLD and the release of Ch were recently observed in rat hippocampal slices [17]. In primary cultures of rat astrocytes, we observed a muscarinic and adrenergic stimulation of PLD activity [8]. All manipulations that cause a rise in the cytoplasmic Ca^{2+} level, e.g., hypoxia and receptor activation (adrenoceptors, NMDA receptor), are expected to enhance the release of Ch via PLA_2 activation [12, 15, 22].

5. Free choline and ACh synthesis

ACh synthesis is dependent on neuronal uptake of Ch from the extracellular space. The extracellular Ch represents a general pool that is maintained by a variety of sources, mainly plasma Ch, free intracellular Ch, Ch-containing phospholipids (hydrolyzed by PLD and PLA_2 ; see above) and ACh (hydrolyzed by ChE). The relative importance of these sources varies depending on the location. Thus ACh hydrolysis and muscarinic mobilization of Ch from phospholipids (see above) are exclusively operating in the cholinergic synapse.

Under normal conditions, the HACU into the cholinergic nerve ending is the rate-limiting step for the synthesis of ACh; inhibition of the HACU by hemicholinium-3 blocks ACh synthesis [26, 28] and release [20]. The extracellular Ch concentration (approximately 4–6 μM), which is above the K_m (1–2 μM) of the HACU, is not rate-limiting unless the synaptic Ch concentration is reduced. On the first glance, it may appear paradoxical that stimulation of ACh release, e.g., by scopolamine [34] or electrical nerve stimulation [24] lowers the extracellular Ch concentration and the ACh tissue content. Stimulation-induced activation of the HACU leads to a decrease in the synaptic Ch level which then may become rate-limiting for the ACh synthesis (see below). In this case, the subsequent reduction of the ACh tissue content can be prevented by exogenous Ch or lecithin (see [35, 36]). Therefore it is plausible that the stimulated, but not the basal ACh release in the rat hippocampus *in vivo* could be enhanced by exogenous Ch and by nicotinamide [18, 19]; nicotinamide mobilizes endogenous Ch from phospholipids [7]. Likewise it was shown that a phospholipase D-catalyzed hydrolysis of phosphatidylcholine can provide Ch as precursor to ACh synthesis [21].

According to the above described mechanisms of brain Ch homeostasis and to previous data [40], exogenous Ch increases the ACh release by enlarging

the pool of surplus bound Ch and the availability of bound Ch as precursor of ACh synthesis. The availability of Ch from phosphatidylcholine can be enhanced by ACh itself through muscarinic receptor activation in the sense of a positive feedback control of ACh synthesis [13].

In the absence of a cholinesterase inhibitor, the extracellular level of Ch showed a biphasic change in response to cholinergic nerve stimulation, namely an initial peak due to hydrolysis of the released ACh followed by a depletion caused by activation of the HACU (see [24, 28]; see above). The response of the extracellular Ch level to cholinesterase inhibition is difficult to predict, especially for a long-lasting neuronal activity: stimulation of the HACU is opposed by the muscarinic release of Ch from phospholipids (see above).

6. Concluding remarks on the synaptic concentrations of choline

Although absolute concentrations of Ch and ACh within the synaptic cholinergic compartment are difficult to monitor, the following conclusions seem justified (for details see above).

1. Under resting conditions, the synaptic (extracellular) concentration of Ch is approximately 4–6 μM , i.e., when ACh is not released to a significant extent and the general homeostatic mechanisms determine the extracellular Ch concentration. In this situation the source of the extracellular Ch is mainly phosphatidylcholine, which represents an almost inexhaustible pool for free Ch. This Ch pool is subject to a variety of receptor-regulated mechanisms. In the absence of ChE inhibition, the efflux of ACh from rat cortex into the microdialysate *in vivo* was 0.28 fmol/min and that of Ch 330 fmol/min [33]. Inhibition of the ChE activity by 80% enhanced the ACh efflux to 1.3 fmol/min (> 700%), which corresponds to a small fraction of the extracellular Ch.

2. Stimulation of cholinergic nerves for a short period leads to a biphasic change of the synaptic Ch level, a brief increase (parallel to the rise of ACh) and a longer-lasting decrease due to neuronal uptake. The decline occurs at the sites of the HACU which are located within the synaptic compartment. During prolonged neuronal activation the decrease of synaptic Ch is dominating, apparently because the Ch originating from ACh hydrolysis is only partially recycled and is eliminated by the homeostatic mechanisms of brain Ch to a significant extent.

3. The idea that Ch may serve as a muscarinic and/or nicotinic agonist has been proposed since several decades. The above kinetic considerations make such a physiological role for the weak agonist

Ch unlikely. It is obvious that Ch cannot cumulate within the synaptic or extrasynaptic compartments. However, it cannot be totally excluded that the synaptic concentration of Ch rises for milliseconds at the ACh release sites to a significant extent and stimulates receptors located in the immediate vicinity [1]. The assumption that ChE inhibition may worsen the symptoms of cholinergic dysfunctions present in Alzheimer's disease, because allegedly the synaptic concentration of Ch is reduced [32], is not compatible with the homeostatic control of brain Ch and the beneficial effects of the ChE inhibitors used for the treatment of this disease.

4. The kinetic and activity profiles of Ch are typical for a biosynthetic nutrient precursor to a neurotransmitter. The brain is protected from a precursor excess by homeostatic mechanisms. However, under certain experimental, pathophysiological and pharmacological conditions Ch supplementation may enhance the release of ACh and may have discrete effects on certain membrane properties such as the density of cholinergic or non-cholinergic receptors [6, 30].

Acknowledgment

This article is based on experimental work supported by the Deutsche Forschungsgemeinschaft.

References

- [1] Alkondon M., Pereira E.D.F., Cortes W.S., Maelicke A., Albuquerque E.X., Choline is a selective agonist of $\alpha 7$ nicotinic acetylcholine receptors in the rat brain neurons, *Eur. J. Neurosci.* 9 (1997) 2734–2742.
- [2] Brehm R., Lindmar R., Löffelholz K., Muscarinic mobilization of choline in rat brain in vivo as shown by the cerebral arterio-venous difference of choline, *J. Neurochem.* 48 (1987) 1480–1485.
- [3] Cohen E.L., Wurtman R.J., Brain acetylcholine: control by dietary choline, *Science* 191 (1976) 561–562.
- [4] Cornford E.M., Braun L.D., Oldendorf W.H., Carrier-mediated blood-brain barrier transport of choline and certain choline analogues, *J. Neurochem.* 30 (1978) 299–308.
- [5] Corradetti R., Lindmar R., Löffelholz K., Mobilization of cellular choline by stimulation of muscarinic receptors in isolated chicken heart and rat cortex in vivo, *J. Pharmacol. Exp. Ther.* 226 (1983) 826–832.
- [6] Couter J.B., Cawley G., Wecker L., Dietary choline supplementation increases the density of nicotine binding sites in rat brain, *J. Pharmacol. Exp. Ther.* 262 (1992) 1128–1132.
- [7] Erb C., Klein J., Enhancement of brain choline levels by nicotineamide: mechanism of action, *Neurosci. Lett.* 249 (1998) 1–4.
- [8] Gonzalez R., Löffelholz K., Klein J., Adrenergic activation of phospholipase D in primary rat astrocytes, *Neurosci. Lett.* 219 (1996) 53–56.
- [9] Kilbinger H., Kruehl M.R., Choline inhibits acetylcholine release via presynaptic muscarinic receptors, *Naunyn-Schmiedeberg's Arch. Pharmacol.* 316 (1981) 131–134.
- [10] Klein J., Köppen A., Löffelholz K., Small rises in plasma choline reverse the negative arteriovenous difference of brain choline, *J. Neurochem.* 55 (1990) 1231–1236.
- [11] Klein J., Köppen A., Löffelholz K., Schmitthenner J., Uptake and metabolism of choline after acute choline administration, *J. Neurochem.* 58 (1992) 870–876.
- [12] Klein J., Holler T., Cappel E., Köppen A., Löffelholz K., Release of choline from rat brain under hypoxia: contribution from phospholipase A₂ but not from phospholipase D, *Brain Res.* 630 (1993) 337–340.
- [13] Klein J., Chalifa V., Liscovitch M., Löffelholz K., Role of phospholipase D in nervous system physiology and pathophysiology, *J. Neurochem.* 65 (1995) 1445–1455.
- [14] Klein J., Lindmar R., Löffelholz K., Muscarinic activation of phosphatidylcholine hydrolysis, *Prog. Brain Res.* 109 (1996) 201–208.
- [15] Klein J., Chatterjee S.S., Löffelholz K., Phospholipid breakdown and choline release under hypoxic conditions: inhibition by bilobalid, a constituent of Ginkgo biloba, *Brain Res.* 755 (1997) 347–350.
- [16] Klein J., Köppen A., Löffelholz K., Regulation of free choline in rat brain: dietary and pharmacological manipulations, *Neurochem. Int.* 32 (1998) 479–485.
- [17] Klein J., Vakil M., Bergman F., Holler T., Iovino M., Löffelholz K., Glutamatergic activation of hippocampal phospholipase D: postnatal fading and receptor desensitization, *J. Neurochem.* 70 (1998) 1679–1685.
- [18] Köppen A., Klein J., Holler T., Löffelholz K., Synergistic effect of nicotineamide and choline administration on extracellular choline levels in the brain, *J. Pharmacol. Exp. Ther.* 266 (1993) 720–725.
- [19] Köppen A., Klein J., Erb C., Löffelholz K., Acetylcholine release and choline availability in rat hippocampus: effects of exogenous choline and nicotineamide, *J. Pharmacol. Exp. Ther.* 282 (1997) 1139–1145.
- [20] Koshimura K., Miwa S., Lee K., Hayashi Y., Hasegawa H., Hamahata K., Fujiwara M., Kimura M., Itokawa Y., Effects of choline administration on in vivo release and biosynthesis of acetylcholine in the rat striatum as studied by in vivo brain microdialysis, *J. Neurochem.* 54 (1990) 533–539.
- [21] Lee H.C., Fellenz M.P., Liscovitch M., Blusztajn J.K., A phospholipase D-catalyzed hydrolysis of phosphatidylcholine provides the choline precursor for acetylcholine synthesis in a human neuronal cell line, LA-N-2, *Proc. Natl. Acad. Sci. USA* 90 (1993) 10086–10090.
- [22] Lindmar R., Löffelholz K., Sandmann J., The release of choline from phospholipids mediated by β -adrenoceptor activation in isolated hearts, *Naunyn-Schmiedeberg's Arch. Pharmacol.* 334 (1986) 228–233.
- [23] Lindmar R., Löffelholz K., Sandmann J., On the mechanism of muscarinic hydrolysis of choline phospholipids in the heart, *Biochem. Pharmacol.* 37 (1988) 4689–4695.
- [24] Löffelholz K., Release of acetylcholine in the isolated heart, *Am. J. Physiol.* 240 (1981) H431–440.
- [25] Löffelholz K., Receptor-regulation of choline phospholipid hydrolysis: a novel source of diacylglycerol and phosphatidic acid, *Biochem. Pharmacol.* 38 (1989) 1543–1549.
- [26] Löffelholz K., Lindmar R., Weide W., Isolated perfused heart and its parasympathetic neuroeffector junction. in: Hanin I., Goldberg A.M. (Eds.), *Progress in Cholinergic Biology: Mo-*

- del Cholinergic Synapses, Raven Press, New York, 1982, pp. 95-111.
- [27] Löffelholz K., Klein J., Köppen A., Choline, a precursor of acetylcholine and phospholipids in the brain, *Progr. Brain Res.* 98 (1993) 199-202.
- [28] MacIntosh F.C., Are choline levels related to acetylcholine release?, in: Barbeau A., Growdon J.H., Wurtman R.J. (Eds.), *Nutrition and the Brain*, Vol. 5, Raven Press, New York, 1979, pp. 201-217.
- [29] Massarelli R., Mykita S., Sorrentino G., The supply of choline to glial cells, in: Fedoroff S., Vernadakis A., (Eds.), *Astrocytes*, Academic Press, New York, 1986, pp. 155-178.
- [30] Miller L.G., Dietary choline alteration. Implications for γ -aminobutyric acid and other neurotransmitter receptors, *Biochem. Pharmacol.* 40 (1990) 1179-1182.
- [31] Papke R.L., Bencherif M., Lippiello P., An evaluation of neuronal nicotinic acetylcholine receptor activation by quaternary nitrogen compounds indicates that choline is selective for the $\alpha 7$ subtype, *Neurosci. Lett.* 213 (1996) 201-204.
- [32] Pereira E.F.R., Alkondon M., Maelicke A., Albuquerque E.X., Functional diversity of nicotinic acetylcholine receptors in the mammalian central nervous system: physiological relevance, in: Arneric S.P., Brioni J.D. (Eds.), *Neuronal Nicotinic Receptors: Pharmacology and Therapeutic Opportunities*, John Wiley and Sons, Inc, 1998, in press.
- [33] Testylier G., Dykes R.W., Acetylcholine release from frontal cortex in the waking rat measured by microdialysis without acetylcholinesterase inhibitors: effects of diisopropylfluorophosphate, *Brain Res.* 740 (1996) 307-315.
- [34] Toide K., Arima T., Effects of cholinergic drugs on extracellular levels of acetylcholine and choline in rat cortex, hippocampus and striatum studied by brain dialysis, *Eur. J. Pharmacol.* 173 (1989) 133-141.
- [35] Tuček S., Problems in the organization and control of acetylcholine synthesis in brain neurons, *Prog. Biophys. Mol. Biol.* 44 (1984) 1-46.
- [36] Tuček S., Short-term control of the synthesis of acetylcholine, *Prog. Biophys. Mol. Biol.* 60 (1993) 59-69.
- [37] Ulus I.H., Millington W.R., Buyukuysal R.L., Kiran B.K., Choline as an agonist: determination of its agonist potency on cholinergic receptors, *Biochem. Pharmacol.* 37 (1988) 2747-2755.
- [38] Vaca K., Pilar G., Mechanisms controlling choline transport and acetylcholine synthesis in motor nerve terminals during electrical stimulation, *J. Gen. Physiol.* 73 (1979) 605-628.
- [39] Vogelsberg V., Neff N.H., Hadjiconstantinou M., Cyclic AMP-mediated enhancement of high-affinity choline transport and acetylcholine synthesis in brain, *J. Neurochem.* 68 (1997) 1062-1070.
- [40] Wecker L., Cawley G., Rothermel S., Acute choline supplementation in vivo enhances acetylcholine synthesis in vitro when neurotransmitter release is increased by potassium, *J. Neurochem.* 52 (1989) 568-575.
- [41] Wuttke W.A., Pentreath V.W., Evidence for the uptake of neuronally derived choline by glial cells in the leech central nervous system, *J. Physiol. (Lond.)* 420 (1990) 387-408.
- [42] Zeisel S.H., Dietary choline: biochemistry, physiology, and pharmacology, *Annu. Rev. Nutr.* 1 (1981) 95-121.

Positive effects of allosteric modulators on the binding properties and the function of muscarinic acetylcholine receptors

Stanislav Tuček^a, Jan Jakubík^a, Vladimír Doležal^a, Esam E. El-Fakahany^b

^a*Institute of Physiology AV CR, Videnská 1083, 14220 Prague, Czech Republic*

^b*University of Minnesota Medical School, Minneapolis, 55455 MN, USA*

Abstract — Data are reviewed indicating that allosteric modulators can enhance the affinities of muscarinic receptors for their antagonists and agonists, that the enhancement of the affinity for agonists is relevant functionally, and that the allosterically induced conformational change also affects the interaction between the receptors and the G proteins. (©Elsevier, Paris)

Résumé — Les effets positifs des modulateurs allostériques sur les propriétés de liaison et la fonction des récepteurs muscariniques de l'acétylcholine. Les modulateurs allostériques peuvent augmenter l'affinité des récepteurs muscariniques pour leurs antagonistes et leurs agonistes. L'augmentation d'affinité pour les agonistes est fonctionnellement significative. Le changement conformationnel, induit allostériquement, modifie l'interaction entre les récepteurs et les protéines G. (©Elsevier, Paris)

muscarinic receptors / presynaptic receptors / allosteric regulation / alcuronium / acetylcholine

1. Introduction

It has been known for more than two decades that certain neuromuscular blockers act as antagonists at muscarinic receptors. In a now classical study, Clark and Mitchelson [2] have shown that the antagonistic action of gallamine on muscarinic receptors in guinea-pig heart atria is allosteric, and Stockton et al. [25] demonstrated the allosteric nature of the action of gallamine by analyzing its effects on the binding of N-[³H]methylscopolamine ([³H]NMS) to cell membranes. Data on the inhibition of muscarinic receptors by neuromuscular blockers have been reviewed by Lee and El-Fakahany [16].

We have discovered that a neuromuscular blocker (alcuronium) can enhance (rather than diminish) the binding of a classical muscarinic ligand ([³H]NMS) to muscarinic receptors in the heart, cerebellum and ileum, and that it does so by increasing the affinity of the receptors for the classical ligand [21, 28]. Our observations demonstrated unequivocally that the allosteric ligand binds to the receptor simultaneously with the classical ligand, and suggested that a new way may exist of how to enhance signal transmission at muscarinic synapses. Subsequent work in which we became involved developed in four main directions: i) allosteric enhancement of the affinity of muscarinic receptors for their antagonists; ii) location of the allosteric binding site; iii) allosteric enhancement of the affinity of muscarinic receptors for their agonists and its functional relevance; and iv) allosteric modulation of the interaction between the receptors and the G proteins (allosteric activation of

the receptors). The following is a short overview of our findings.

2. Allosteric enhancement of the affinity for antagonists

Alcuronium-induced enhancement of the affinity of muscarinic receptors for [³H]NMS is subtype specific and only occurs at the M₂ and M₄ receptor subtypes. The affinities of the M₁, M₃ and M₅ receptor subtypes for [³H]NMS are diminished by alcuronium, and so are the affinities of all receptor subtypes for [³H]quinuclidinyl benzilate ([³H]QNB) [9, 11, 28]. The difference between the effects of alcuronium on the affinities for [³H]NMS and [³H]QNB cannot be explained by the presence of quarternary nitrogen in the former and its absence from the latter antagonist because alcuronium also enhances the affinity of the M₂ receptors for [³H]atropine (a compound without a quarternary nitrogen) and diminishes their affinity for N-[³H]methyl-QNB (a compound with a quarternary nitrogen) [6]. Cellular components other than the muscarinic receptors are probably not necessary for the enhancement of the affinity for [³H]NMS since the phenomenon can also be observed on solubilized receptors uncoupled from G proteins [19].

Other compounds with the potential to allosterically enhance the binding of ligands to the classical (orthosteric) binding sites of muscarinic receptors include strychnine [15, 23], brucine [1, 13], eburnamonine [13, 24], vincamine [13], fangchinoline and tetrandrine [4] and 9-methoxy- α -lapachone [5]. It

has not been clarified which features of the molecule of the allosteric modulator are important for its positive cooperative effect with regard to the binding of orthosteric ligands. In fact, no allosteric modulator of muscarinic receptors is known which would produce only positive effects. The direction of the interaction (positive or negative) varies depending on the modulator, the orthosteric ligand, and the subtype of the receptor.

3. Location of the allosteric binding site

Alcuronium and other allosteric modulators of muscarinic receptors slow down both the association of ligands with and their dissociation from the orthosteric binding sites [7, 20]. Data on the effects of alcuronium on the kinetics of [^3H]NMS binding can be explained on the assumption that alcuronium (when attached to the allosteric binding site) hinders both the access of [^3H]NMS to and its departure from the orthosteric binding site [22]. This led to the proposal that the binding site for alcuronium is located close to but more extracellularly than the orthosteric site, perhaps at the entry to the binding pocket of muscarinic receptors [22]. The proposed location of the allosteric binding site received support from experiments with site-directed mutations of muscarinic receptors [17, 18] and with chemical modification of their functionally important amino acid residues [8, 10]. Allosteric modifiers protect the tyrosine and aspartate residues in the area of the orthosteric binding site against chemical modification, apparently by hindering the access of the modifiers to the orthosteric site [8]. By using chemical modifications, it proved possible to separate the action of alcuronium on the kinetics of [^3H]NMS binding and on the affinity of receptors for [^3H]NMS, which indicates a degree of independence of these two actions of alcuronium and other allosteric ligands [10]. Allosteric modulators with positive and negative effects on the affinity for [^3H]NMS compete for the same binding domain [23].

4. Allosteric enhancement of the affinity for agonists and its functional relevance

The effects of allosteric ligands on the binding of muscarinic receptor agonists were little studied because the affinity for agonists is generally lower than that for the antagonists and it is difficult to utilize the conventional radioligand binding methods. We applied an indirect approach and investigated the effects of five allosteric modulators on the affinities of the M_1 - M_4 muscarinic receptors for twelve mus-

carinic agonists [13]. We have found that the affinity for acetylcholine is enhanced by brucine at the M_1 and M_3 receptors and by eburnamonine at the M_2 and M_4 receptors. In general, the allosteric enhancement of the affinity for various agonists was low. It was the highest for the combination of eburnamonine and pilocarpine at the M_2 receptors (25-fold). Allosteric enhancement of the affinity for acetylcholine by brucine and its derivatives was also reported by Birdsall et al. [1].

To detect whether the increase in the affinity for muscarinic agonists detected in radioligand binding experiments is of functional relevance, we performed experiments in which we measured the inhibitory effect of muscarinic receptor agonists (acting on the presynaptic muscarinic receptors) on the release of acetylcholine from rat striatal slices. We discovered that the inhibitory actions of furmethide, oxotremorine- M and bethanechol were potentiated by brucine, in accordance with what could be expected from data on the allosteric interaction between the binding of brucine and the three agonists to the M_4 receptors [3].

5. Allosteric activation of muscarinic receptors

Allosteric modulators induce a change in the conformation of the orthosteric binding site, which results in the change of the affinity for the orthosteric ligands. It is a question whether the change of conformation only concerns the area of the orthosteric site, or other receptor domains just as well. We have found in experiments on CHO cells expressing individual subtypes of muscarinic receptors that the allosteric modulators, acting in the absence of muscarinic agonists, had agonist-like effects (inhibition of the synthesis of cyclic AMP in cells expressing the M_2 and M_4 receptors, and stimulation of the production of inositol phosphates in cells expressing the M_1 and M_3 receptors) [12]. These effects were not observed in cells which had not been transfected with the genes for muscarinic receptors, and were not blocked by QNB (indicating that the modulators did not bind to the orthosteric binding site). It is thus apparent that muscarinic receptors can be activated not only from their orthosteric, but also from their allosteric binding sites. The activating effect could be also observed in a reconstituted system containing purified M_2 receptors and purified Go proteins. Data obtained in the reconstituted system indicate that receptor activation induced by the allosteric ligands differs from that induced by the orthosteric ligands [14].

6. Conclusion

The discovery of positive cooperativity between the allosteric and orthosteric ligands of muscarinic receptors opens new ways to pharmacological modulation of neurotransmission on muscarinic synapses and adds to the understanding of the molecular physiology of muscarinic receptors [26, 27].

Acknowledgments

Our work was supported by the Grant Agencies of the Czech Republic and of the Academy of Sciences of the Czech Republic, and by Fogarty International Research Collaboration Award.

References

- [1] Birdsall N.J.M., Farries T., Gharagozloo P., Kobayashi S., Kuonen D., Lazareno S., Popham A., Sugimoto M., Selective allosteric enhancement of the binding and actions of acetylcholine at muscarinic receptor subtypes, *Life Sci.* 60 (1997) 1047–1052.
- [2] Clark A.L., Mitchelson F., The inhibitory effect of gallamine on muscarinic receptors, *Br. J. Pharmacol.* 58 (1976) 323–331.
- [3] Doležal V., Tuček S., The effects of brucine and alcuronium on the inhibition of [³H]acetylcholine release from rat striatum by muscarinic receptor agonists, *Br. J. Pharmacol.* (1998), in press.
- [4] Guo Z.D., Kameyama K., Rinken A., Haga T., Ligand binding properties of muscarinic acetylcholine receptor subtypes (m1–m5) expressed in baculovirus-infected insect cells, *J. Pharmacol. Exp. Ther.* 274 (1995) 378–384.
- [5] Guo Z.D., Haga T., Itokawa H., Mizobe F., Allosteric binding of 9-methoxy- α -lapachone and alcuronium to the muscarinic acetylcholine receptor m2 subtype, *Biomed. Res.* 16 (1995) 327–335.
- [6] Hejnová L., Tuček S., El-Fakahany E.E., Positive and negative allosteric interactions on muscarinic receptors, *Eur. J. Pharmacol.* 291 (1995) 427–430.
- [7] Holzgrabe U., Mohr K., Allosteric modulation of ligand binding to muscarinic acetylcholine receptors, *Drug Dev. Today* 3 (1998) 214–222.
- [8] Jakubík J., Tuček S., Protection by alcuronium of muscarinic receptors against chemical inactivation and location of the allosteric binding site for alcuronium, *J. Neurochem.* 63 (1994) 1932–1940.
- [9] Jakubík J., Tuček S., Two populations of muscarinic binding sites in the chick heart distinguished by affinities for ligands and selective inactivation, *Br. J. Pharmacol.* 113 (1994) 1529–1537.
- [10] Jakubík J., Tuček S., Positive allosteric interactions on cardiac muscarinic receptors: effects of chemical modifications of disulphide and carboxyl groups, *Eur. J. Pharmacol.* 289 (1995) 311–319.
- [11] Jakubík J., Bačáková L., El-Fakahany E.E., Tuček S., Subtype selectivity of the positive allosteric action of alcuronium at cloned M₁–M₅ muscarinic acetylcholine receptors, *J. Pharmacol. Exp. Ther.* 274 (1995) 1077–1083.
- [12] Jakubík J., Bačáková L., Lisá V., El-Fakahany E.E., Tuček S., Activation of muscarinic acetylcholine receptors via their allosteric binding sites, *Proc. Natl. Acad. Sci. USA* 93 (1996) 8705–8709.
- [13] Jakubík J., Bačáková L., El-Fakahany E.E., Tuček S., Positive cooperativity of acetylcholine and other agonists with allosteric ligands on muscarinic acetylcholine receptors, *Mol. Pharmacol.* 52 (1997) 172–179.
- [14] Jakubík J., Haga T., Tuček S., Allosteric ligand-receptor-G protein interactions in lipid vesicles, *Life Sci.* 60 (1997) 1170.
- [15] Lazareno S., Birdsall N.J.M., Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors, *Mol. Pharmacol.* 48 (1995) 362–378.
- [16] Lee N.H., El-Fakahany E.E., Allosteric antagonists of the muscarinic acetylcholine receptor, *Biochem. Pharmacol.* 42 (1991) 199–205.
- [17] Leppik R.A., Miller R.C., Eck M., Paquet J.-L., Role of acidic amino acids in the allosteric modulation by gallamine of antagonist binding at the m2 muscarinic acetylcholine receptor, *Mol. Pharmacol.* 45 (1994) 983–990.
- [18] Matsui H., Lazareno S., Birdsall N.J.M., Probing of the location of the allosteric site on m1 muscarinic receptors by site-directed mutagenesis, *Mol. Pharmacol.* 47 (1995) 88–98.
- [19] Musílková J., Tuček S., Positive allosteric action of alcuronium on solubilized cardiac muscarinic receptors, *Neurochem. Int.* 27 (1995) 337–343.
- [20] Nedoma J., Tuček S., Danilov A.F., Shelkovnikov S.A., Stabilization of antagonist binding to cardiac muscarinic acetylcholine receptors by gallamine and other neuromuscular blocking drugs, *J. Pharmacol. Exp. Ther.* 236 (1986) 219–223.
- [21] Nedoma J., Tuček S., Shelkovnikov S., Danilov A., Allosteric effects of gallamine and alcuronium on muscarinic receptors, in: Tuček S. (Ed.), *Synaptic Transmitters and Receptors*, Academia, Praha, 1987, pp. 108–112.
- [22] Proška J., Tuček S., Mechanisms of steric and cooperative actions of alcuronium on cardiac muscarinic acetylcholine receptors, *Mol. Pharmacol.* 45 (1994) 709–717.
- [23] Proška J., Tuček S., Competition between positive and negative allosteric effectors on muscarinic receptors, *Mol. Pharmacol.* 48 (1995) 696–702.
- [24] Proška J., Tuček S., Positive allosteric action of eburnamine on cardiac muscarinic acetylcholine receptors, *Eur. J. Pharmacol.* 305 (1996) 201–205.
- [25] Stockton J.M., Birdsall N.J.M., Burgen A.S.V., Hulme E.C., Modification of the binding properties of muscarinic receptors by gallamine, *Mol. Pharmacol.* 23 (1983) 551–557.
- [26] Tuček S., Is the R and R* dichotomy real?, *Trends Pharmacol. Sci.* 18 (1997) 414–416.
- [27] Tuček S., Proška J., Allosteric modulation of muscarinic acetylcholine receptors, *Trends Pharmacol. Sci.* 16 (1995) 205–212.
- [28] Tuček S., Musílková J., Nedoma J., Proška J., Shelkovnikov S., Vorlíček J., Positive cooperativity in the binding of alcuronium and N-methylscopolamine to muscarinic acetylcholine receptors, *Mol. Pharmacol.* 38 (1990) 674–680.

On the transcriptional regulation of neuronal nAChR genes

Jean-Marc Matter, Lidia Matter-Sadzinski, Tomas Roztocil, Marie-Clemencia Hernandez, Sabine Couturier, Ming-Thong Ong, Marc Ballivet

Department of Biochemistry, University of Geneva, 30, quai Ernest Ansermet, CH-1211 Geneva 4, Switzerland

Abstract — The promoters driving transcription of the neuronal nicotinic genes $\alpha 7$ and $\beta 3$ have been characterized in the chicken. Although their regulatory modalities are thoroughly different, they nevertheless lead to co-expression in the same neurons. (©Elsevier, Paris)

Résumé — La régulation transcriptionnelle des gènes nicotiniques neuronaux. Les promoteurs qui contrôlent la transcription des gènes nicotiniques neuronaux $\alpha 7$ et $\beta 3$ ont été caractérisés chez le Poulet. Quoique leurs modalités régulatrices diffèrent profondément, elles aboutissent cependant à leur co-expression dans les mêmes neurones. (©Elsevier, Paris)

nicotinic receptors / genetics / physiology / gene expression regulation

1. Introduction

Eleven genes encoding neuronal nicotinic acetylcholine receptors (nAChR) subunits have been identified in vertebrates (for review see [14, 16], and closely related neuronal nAChR genes have been characterized in invertebrate model systems such as *Drosophila melanogaster* (for review see [6] and *Caenorhabditis elegans* [1, 5, 18]. They fall into homology classes that predate the divergence of vertebrates and invertebrates, demonstrating that they have been present as families of related genes since a very early stage in nervous system evolution [10, 13]. Although the availability of cloned neuronal nAChRs has led to a massive improvement in our understanding of their physiology and structure, very little is known about their functions in the central nervous system, and the reason for their multiplicity remains a puzzle. There is evidence that they are mostly, but not exclusively, effectors in the presynaptic control of neurotransmitter release [19]. Their role in nociception [2, 8] and their implication in at least one form of familial epilepsy [17] suggest that they play varied and diversified parts in neural function.

One plausible view has it that the ancestral nAChRs were functional homopentamers, as exemplified by the $\alpha 7$ – $\alpha 9$ series of present-day subunits. Such structures are inherently limited in the number of different regulatory interactions they can accommodate, so that evolution retained variants of duplicated genes whose products were able to assemble into heteropentamers of various, non-equivalent compositions. Heteropentamers are much richer in potential regulatory sites than homopentamers be-

cause of the vastly increased repertoire of distinct subunit interfaces they provide. In addition to increasing the range of ligands capable of modulatory activities on an increased spectrum of receptor types, heteropentamery also allowed for more modulators to interact with a single receptor species, thereby increasing the range of possible coincidence detectors, a most desirable property in signaling integration [3].

As the repertoire of nAChR subunits increased by gene duplication and by selection of variants capable of novel interactions with ligands, it is likely that the promoters governing the transcription of individual nAChR genes were also submitted to evolutionary pressure tending to select and retain a variety of different transcriptional modalities. Such a process would have the decisive advantage of earmarking subsets of nAChR genes for particular uses in different tissues (e.g., muscle vs. neuronal receptors), or at different developmental stages (e.g., embryonic vs. adult muscle receptors).

In our laboratory, we have recently examined the promoter regions and associated transcription factors that regulate the activity of the neuronal homomeric $\alpha 7$ and heteromeric $\beta 3$ nAChR genes. We were prompted to select these genes not only because they are respective examples of the homomeric and heteromeric nAChRs, but also because whereas $\alpha 7$ is widely expressed very early on in neuronal and non-neuronal precursors [4, 9, 12], later to acquire a more restricted expression domain, $\beta 3$ has a narrowly restricted expression pattern at all stages of development [7, 11]. We have found that although the two genes are regulated by thoroughly different *cis-trans* interactions, their expression domains overlap in the ganglion cells of the developing retina. These

observations illustrate the diversity and versatility of the regulatory mechanisms that have evolved in parallel with an increasing nAChR gene repertoire.

2. Materials and methods

To determine which region of a cloned nAChR gene does contain all or most of the information required for proper spatial and temporal expression, we examined the DNA sequences located immediately 5' of the transcription initiation sites. This was achieved by subcloning upstream of reporter genes such as the bacterial β -galactosidase (with added nuclear localization signal) and chloramphenicol acetyltransferase (CAT) genes, transfecting into a range of freshly dissociated neural and non-neural cells from tissues isolated at various developmental stages, and scoring for reporter gene activity. The β -galactosidase reporter enabled us to detect which cell types were capable of activating the promoter, whereas the CAT reporter afforded an easy quantitative assay of the overall activity of a given tissue. This experimental approach is dependent on the standardized, efficient and reproducible lipofectin-mediated transfection procedure we have developed in the laboratory. Properly used, the technique permits efficient transfection of many types of neural (neuroretina, optic tectum, telencephalon, spinal cord, peripheral ganglia, etc.) and non-neural tissues at all developmental stages [12]. Transfection efficiency reaches 30%, and transfection-induced neuronal death appears minimal.

3. Results and discussion

3.1. The $\alpha 7$ promoter

A short DNA fragment, 177 base pairs (bp) in length and encompassing the two major transcription cap sites, contains essentially all the information re-

quired for tissue and stage specific control [12]. In the retina, the 177-bp fragment drives reporter gene transcription in the same cells (i.e., the ganglion cells) and at the same developmental stages as the endogenous $\alpha 7$ gene.

The $\alpha 7$ promoter sequence is extremely rich in the bases G and C and is therefore a candidate for multiple regulatory interactions with transcription factors such as SP1, AP2 and GCF, whose consensus binding sites are GC-rich (figure 1A). Site-directed mutagenesis either to enhance or to abolish promoter activity is in progress and preliminary results indicate that multiple point mutations are required for promoter inactivation, suggesting that multiple, overlapping binding sites intervene to provide a degree of redundancy (S. Couturier, unpublished results). We do not know as yet if the tissue and stage-specific regulation of transcription is achieved by dedicated, nervous system specific transcription factors or by a cell-type specific combination and/or ratio of ubiquitous transcription factors.

3.2. The $\beta 3$ promoter

Analysis of the regulatory sequence directing $\beta 3$ gene transcription has enabled us to isolate a DNA fragment, 143 bp in length and located immediately upstream of the transcription cap site, containing all the cis-elements required for specifically targeting reporter gene expression to certain neurons in the retina, including ganglion cells and a subset of amacrine neurons (figure 1B). In addition, the 143-bp DNA fragment has proper temporal and tissue resolution, being able to promote stronger reporter trans-

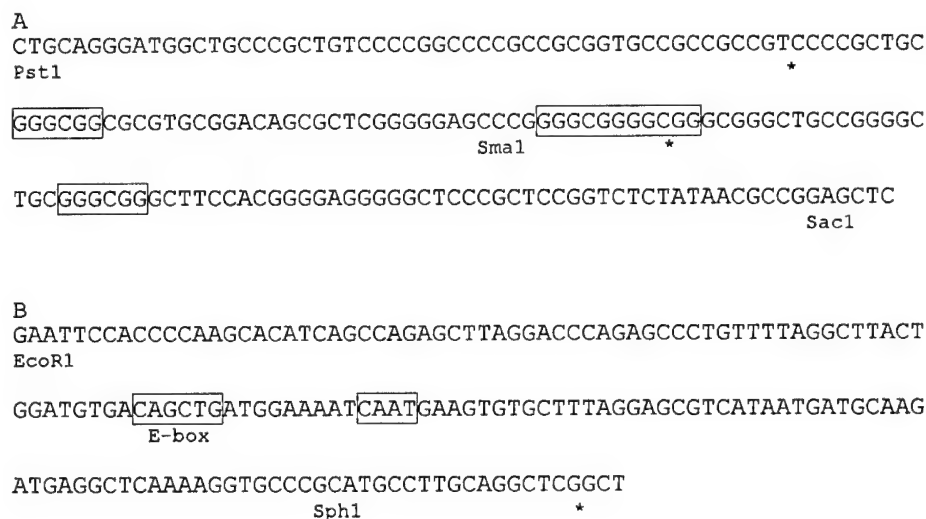


Figure 1. Sequences of the $\alpha 7$ (A) and $\beta 3$ (B) promoters. The major sites of transcription initiation are marked by an asterisk. The boxed motifs are SP1 consensus sequences in A and E- and CAAT-boxes in B.

cription in E5 than in E13 retina, and failing to sustain transcriptional activity in optic tectum neurons or in glia [7, 11].

Mutagenesis of the several potential consensus binding sites for transcriptional factors within the 143-bp control element has revealed that two of them, an E-box and a nearby CAAT-box, are critical for function. E-boxes are the binding sites for basic helix-loop-helix (bHLH) transcription factors, many of which participate in neural determination and differentiation. Accordingly, we attempted to transactivate the transfected $\beta 3$ promoter by supplying cloned neural bHLH proteins to cells that normally do not express $\beta 3$.

We found that although none of the tested neural bHLH proteins (ash1, CTF4, neuroD and neuroM) were able to activate the $\beta 3$ promoter, the muscle bHLH factor myoD did turn on transcription at the transfected (and endogenous) $\beta 3$ promoter in a range of different neurons, but not in non-neural cell types. Moreover, we demonstrated that the ability of the $\beta 3$ promoter to discriminate between such related bHLH factors as myoD and neuroD is due to the short 70 bp sequence extending 3' of the E-box, whereas the elements placed in 5' of the E-box are entirely dispensable. In marked contrast, the promoter of the muscle nAChR receptor $\alpha 1$ subunit was found to be activatable by all tested bHLH proteins in neurons, and exclusively by myoD in non-neural cells [15].

One possible interpretation of these findings is that, whereas myoD is able to activate the $\alpha 1$ promoter as a homodimer (or perhaps as a heterodimer with one of the ubiquitous bHLH proteins), it must form a heterodimer with a neural bHLH to activate the $\beta 3$ promoter. In contrast, none of the tested neural bHLH factors are capable of activating $\beta 3$, either as homodimers or as heterodimers with resident bHLH factors. Thus, we have obtained rigorous proof that a neural bHLH factor of limited host-range is responsible for tissue-specific expression of the $\beta 3$ gene, but that factor has yet to be isolated and identified.

In addition, a CAAT-box located 9 bp downstream of the E-box is also required for promoter activation, and a minimal spacing between the CAAT- and E-boxes must be maintained for proper function. This suggests that activation of the $\beta 3$ promoter requires protein-protein interactions between factors bound at the two sites [15]. Thus, the limited expression domain of the $\beta 3$ gene in the nervous system may simply result from the coincident expression of a bHLH protein and of a CAAT-interacting factor. Both these proteins may have quite large expression domains, as it suffices that they rarely overlap to ensure strin-

gent transcriptional control of the genes they coregulate.

Acknowledgments

We are indebted to Christine Alliod for expert technical assistance. Work in our laboratory was funded by the State of Geneva, by grants from the Swiss National Science Foundation (to JMM and MB) and by a grant from the Sandoz Foundation (to LMS).

References

- [1] Ballivet M., Alliod C., Bertrand S., Bertrand D., Nicotinic acetylcholine receptors in the nematode *Caenorhabditis elegans*, *J. Mol. Biol.* 258 (1996) 261–269.
- [2] Bannon A.W., Decker M.W., Holladay M.W., Curzon P., Donnelly-Roberts D., Puttfarcken P.S., Bitner R.S., Diaz A., Dickenson A.H., Porcino R.D., Williams M., Arneric S.P., Broad-spectrum, non-opioid analgesic activity by selective modulation of neuronal nicotinic acetylcholine receptors, *Science* 279 (1998) 77–81.
- [3] Changeux J.P., Edelstein S., The allosteric model after 30 years, *Neuron* (1998), in press.
- [4] Corriveau R.A., Romano S.J., Conroy W.G., Oliva L., Berg D.K., Expression of neuronal acetylcholine receptor genes in vertebrate skeletal muscle during development, *J. Neurosci.* 15 (1995) 1372–1383.
- [5] Fleming J.T., Squire M.D., Barnes T.M., Tornoe C., Matsuda K., Ahnn J., Fire A., Sulston J.E., Barnard E.A., Sattelle D.B., Lewis J.A., *Caenorhabditis elegans* levamisole resistance genes *lev-1*, *unc-29*, and *unc-38* encode functional nicotinic acetylcholine receptor subunits, *J. Neurosci.* 17 (1997) 5843–5857.
- [6] Gundelfinger E.D., Hess N., Nicotinic acetylcholine receptors of the central nervous system of *Drosophila*, *Biochim. Biophys. Acta* 1137 (1992) 299–308.
- [7] Hernandez M.C., Erkman L., Matter-Sadzinski L., Roztocil T., Ballivet M., Matter J.M., Characterization of the nicotinic acetylcholine receptor beta3 gene. Its regulation is effected by a promoter 143 base pairs in length, *J. Biol. Chem.* 270 (1995) 3224–3233.
- [8] Holladay M.W., Dart M.J., Lynch J.K., Neuronal nicotinic acetylcholine receptors as targets for drug discovery, *J. Med. Chem.* 40 (1997) 4169–4194.
- [9] Howard M.J., Gershon M.D., Margiotta J.F., Expression of nicotinic acetylcholine receptors and subunit mRNA transcripts in cultures of neural crest cells, *Dev. Biol.* 170 (1995) 479–495.
- [10] Le Novère N., Changeux J.P., Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells, *J. Mol. Evol.* 40 (1995) 155–172.
- [11] Matter J.M., Matter-Sadzinski L., Ballivet M., Activity of the beta3 nicotinic receptor promoter is a marker of neuron fate determination during retina development, *J. Neurosci.* 15 (1995) 5919–5928.
- [12] Matter-Sadzinski L., Hernandez M.C., Roztocil T., Ballivet M., Matter J.M., Neuronal specificity of the $\alpha 7$ nicotinic acetylcholine receptor promoter develops during morphogenesis of the central nervous system, *EMBO J.* 11 (1992) 4529–4538.
- [13] Ortells M.O., Lunt G.G., Evolutionary history of the ligand-gated ion-channel superfamily of receptors, *Trends Neurosci.* 18 (1995) 121–127.

- [14] Role L.W., Berg D.K., Nicotinic receptors in the development and modulation of CNS synapses, *Neuron* 16 (1996) 1077-1085.
- [15] Roztocil T., Matter-Sadzinski L., Gomez M., Ballivet M., Matter J.M., Functional properties of the neuronal nicotinic acetylcholine receptor beta3 promoter in the developing central nervous system, *J. Biol. Chem.* 273 (1998), 15131-15137.
- [16] Sargent P.B., The diversity of neuronal nicotinic acetylcholine receptors, *Annu. Rev. Neurosci.* 16 (1993) 403-443.
- [17] Steinlein O.K., Mulley J.C., Propping P., Wallace R.H., Phillips H.A., Sutherland G.R., Scheffer I.E., Berkovic S.F., A missense mutation in the neuronal nicotinic acetylcholine receptor alpha4 subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy, *Nat. Gen.* 11 (1995) 201-203.
- [18] Treinin M., Chalfie M., A mutated acetylcholine receptor subunit causes neuronal degeneration in *C. elegans*, *Neuron* 14 (1995) 871-877.
- [19] Wonnacott S., Presynaptic nicotinic ACh receptors, *Trends Neurosci.* 20 (1997) 92-98.

Position effect variegations and brain-specific silencing in transgenic mice overexpressing human acetylcholinesterase variants

Meira Sternfeld^a, James D. Patrick^b, Hermona Soreq^{a*}

^aDepartment of Biological Chemistry, The Institute of Life Sciences, The Hebrew University of Jerusalem, 91904 Israel

^bDivision of Neuroscience, Baylor College of Medicine, Houston, TX 77030-3498, USA

Abstract — Position effect variegations as well as brain-specific silencing were observed in novel transgenic mouse pedigrees expressing human acetylcholinesterase (AChE) variants. Muscle AChE activities varied between 1.6- and 350-fold of control in these lines, one carrying insertion-inactivated InE6-AChE and two with 'readthrough' I4/E5 AChE, all under control of the ubiquitous CMV promoter. In contrast, brain AChE levels remained within a range of 1.5-fold over control, suggesting an upper limit of brain AChE which is compatible with life. (©Elsevier, Paris)

Résumé — Effets positionnels de transgènes de variants d'acétylcholinestérase humaine et inhibition spécifique de leur expression dans le cerveau de souris transgéniques. Les effets positionnels de transgènes constitués de variants du gène de l'acétylcholinestérase humaine, ainsi que l'inhibition spécifique de leur expression ont été observés dans de nouvelles lignées de souris transgéniques. Les activités de l'acétylcholinestérase (AChE) dans les muscles de ces animaux varient dans un rapport de 1,6 à 350 selon la lignée. Une des lignées comporte une forme inactive de l'AChE (InE6-AChE), deux autres comportent une forme de l'AChE contenant l'intron 4 'readthrough' (I4/E5). Tous les variants de l'AChE utilisés dans ces expériences sont sous le contrôle du promoteur ubiquiste du cytomegalovirus. Par contre, l'activité de l'AChE ne varie que dans un rapport de 1 à 2 dans le cerveau, démontrant qu'il existe une limite supérieure du niveau d'expression de l'AChE compatible avec la survie. (©Elsevier, Paris)

acetylcholinesterase / alternative splicing variants / gene dosage effects / muscle / transgenic pedigrees

1. Introduction

Many studies of gene regulation in mammals *in vivo* have been made possible by transgenic technology (for review see [10]). Early transgenes were constructed with several promoter and enhancer sequences, however, these were rarely sufficient to ensure native levels or tissue-specific expression [19]. Thereafter, the site of genomic integration of mammalian transgenes was found to play a major role in their control [6]. Therefore, individual *Drosophila* or mouse lines with multicopy transgenes exhibited expression levels that were partially or completely unrelated to the number of the integrated transgenes [20]. These phenomena may be especially pronounced when the overexpressed transgene changes a vital process such as cholinergic neurotransmission, for example in transgenic mice carrying the human transgene for the acetylcholine (ACh) hydrolysing enzyme acetylcholinesterase (acetylcholine acetylhy-

drolase, EC3.1.1.7, AChE). In adult transgenic mice overexpressing synaptic AChE in brain neurons [3] we observed structural and functional abnormalities in neuromuscular junctions [1] as well as attenuation of dendrite branching and depletion of dendritic spines harboring synapses of cortical neurons [4]. However, the specific contribution of the transgenic AChE toward each of these phenomena remained unknown.

In addition to its function in cholinergic neurotransmission, compelling evidence demonstrates a neurite growth-promoting activity of AChE. This activity does not depend on the capacity of AChE to hydrolyse ACh, as it persists in the presence of active site inhibitors [11, 17, 24] and is sustained in insertion-inactivated human recombinant AChE (InE6-AChE) [25]. However, the neuritogenic activity of AChE was found to be limited to the synapse-characteristic and membrane-associated E6-AChE isoform which includes the exon 6-encoded amphipathic C-terminus. In contrast, the non-synaptic secretory I4-AChE isoform, which terminates the pseudointron 4-derived hydrophilic peptide, did not affect neurite growth from *Xenopus* motoneurons, nor did it promote process extension from rat glioma cells [12].

Another developmental activity of AChE relates to synapse growth and maintenance. This function

*Correspondence and reprints

Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CMV, cytomegalovirus; Hp, human acetylcholinesterase gene proximal promoter (586 bp upstream from the transcription initiation site); PCR, polymerase chain reaction; SV40, simian virus 40; ATCh, acetylthiocholine; BTCh, butyrylthiocholine.

depends on increasing the enzyme's hydrolytic capacity within the synapse. Evidence supporting this notion is that both E6 AChE [21–23] and a non-natural C-terminally truncated form, E4 AChE, promote the enlargement of *Xenopus* neuromuscular junctions, but the catalytically inactive InE6-AChE and the nonsynaptic AChE isoform I4 AChE do not [25]. However, it remained uncertain whether I4 AChE is also inert during mammalian development.

Two recent developments call for reconsideration of these two questions. First, several active site inhibitors of AChE were approved for chronic therapeutic use in Alzheimer's disease [16]. This implies the continued presence of the catalytically inactive E6-AChE protein, with potential effects on synapse maintenance and plasticity, in brain neurons of treated patients. Second, acute psychological stress and exposure to active-site AChE inhibitors were both shown to induce massive long-term accumulation of I4-AChE in the mammalian brain [13, 14]. Altogether, these called for testing the *in vivo* effects of excess InE6-AChE or I4-AChE on the mammalian brain and muscle. Towards these goals, we now report the creation of transgenic mouse lines carrying these two transgenes and demonstrate position effects of their insertion into the host mouse genome on the extent and tissue specificity of their expression in brain and muscle.

2. Materials and methods

Construction of vectors and the corresponding PCR primers were recently described [25]. To create founder transgenic mice, the I4-AChE and InE6-AChE inserts were both excised from the corresponding plasmids together with the CMV minimal promoter-enhancer sequence and the SV40 polyadenylation signal, using enzymatic restriction with *Kpn*I and *Spe*I. Purified insert DNA was then injected into 70 pronuclei of fertilized mouse eggs for each insert, essentially as previously described [3]. Transgene-carrying mice were identified by PCR analysis of tail DNA and transgene penetrance determined at each generation. To evaluate copy numbers, we performed a semi-quantitative PCR analysis as described previously [13] and in comparison to parallel reactions with plasmid DNA of known concentration.

Xenopus oocyte microinjection and homogenization were as detailed elsewhere [25]. To determine the thermal stability of the variant AChEs, homogenates of *Xenopus* oocytes were incubated at the noted temperatures and times.

Mouse tissues (brain or muscle) were isolated as described elsewhere [3]. Hydrolysis rate of acetylthiocholine (ATCh) was measured in *Xenopus* oocyte or mouse tissue homogenates using an automated multiwell colorimetric assay as described [1, 25]. Protein concentrations were measured using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, 2000 Alfred Nobel Dr., Hercules, CA 94547, USA).

3. Results

To study the regulation of AChE expression in the mammalian brain and muscle, we created a series of transgenic mouse pedigrees overexpressing several variants of human AChE. Four human AChEcDNA constructs were employed for this study. These all included the potent, ubiquitous minimal CMV enhancer-promoter, except for one construct where the proximal 586 base pair fragment from the human AChE gene was used [3]. The coding sequences included two natural variants and one engineered construct. These encode the brain and muscle-abundant E6-AChE isoform carrying the exon 6-derived amphipathic C-terminal peptide [22]; the 'readthrough' I4-AChE isoform C-terminated with the pseudointron I4-derived hydrophilic peptide [12]; and the insertion-inactivated InE6-AChE isoform C-terminated with the exon 6-derived peptide but with no capacity to hydrolyze ACh [25]. Figure 1 presents these different vectors and the distinct properties predicted for their protein products.

We have used plasmid DNA-microinjected *Xenopus* oocytes as an analytical system for the assessment of the expression efficacy of the above vectors. Gel electrophoresis under non-denaturing conditions revealed production and E6-AChE characteristic migration properties for the catalytically inactive but immunopositive InE6-AChE variant [25]. The two catalytically active E6 and I4 AChE variants both retained over 90 and 50% of their hydrolytic capacity in oocyte homogenates when incubated for 5 h at 37 °C and 42 °C, respectively (figure 2). This demonstrated that the variant C-terminal peptides do not contribute significantly toward the heat stability of AChE. That the C-terminally truncated E4-AChE isoform presented similar heat stability, confirmed this conclusion [25].

Novel transgenic FVB/N mouse lines were created with the CMV I4-AChE and the CMV InE6-AChE vectors and their properties compared to those of the HpE6-AChE pedigree [1, 3, 4] and of control FVB/N mice. Each of these transgenes displayed unimpaired Mendelian inheritance patterns for over five generations, suggesting compatibility with survival. Figure 3 presents these pedigrees, one for HpE6-AChE, two with CMV I4-AChE and one with CMV InE6-AChE. Kinetic follow-up of PCR amplifications, using host mouse DNA and primers selective for each of the transgenes revealed variable first cycle of appearance (27, 27, 30 and 30 for 500 ng samples of genomic DNA) for the two independent pedigrees with CMV I4-AChE transgenes, lines 45 and 70, one with HpE6-AChE and one with CMV InE6-AChE, respectively.

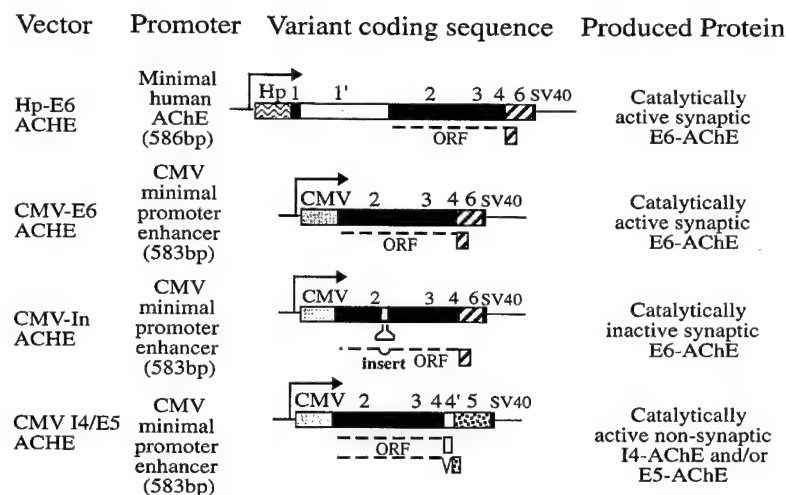


Figure 1. Human AChE variants overexpressed in transgenic model systems. Presented are recombinant DNA inserts from plasmids constructed to perform this transgenic study. Hp, human ACHE minimal promoter; CMV, cytomegalovirus minimal promoter-enhancer; SV40, consensus polyadenylation site. Arrows indicate the orientation of transcription and exons 1–6 are numbered. Intron I1 and pseudointron I4 are hyphenated and the open reading frame of each coding region is noted by a dashed line below it, with the naturally variable C-terminal peptides and the engineered inactivating insert highlighted. The major characteristics of the AChE proteins produced from each of these vectors are also noted. See Beerli et al. [3], Seidman et al. [22] and Sternfeld et al. [25] for further details of each of these vectors.

The efficacy of AChE expression in brain and muscle tissues from these transgenic mice was evaluated by ATCh hydrolysis rates in tissue homogenates in the presence and absence of the specific BChE inhibitor IsoOMPA. These measurements demonstrated drastic differences in the specific activity of muscle AChE (table I). Hydrolysis capacity reached levels of 190 ± 22 (S.E.M.) and 2700 ± 330 (S.E.M.) nmol ATCh hydrolyzed/min/mg protein in the two I4 AChE lines as compared to 7.6 ± 0.5 (S.E.M.), and 12 ± 2.3 (S.E.M.) nmol/min/mg in muscle from control FVB/N mice and transgenics carrying InE6-AChE, respectively.

In contrast to the drastic variabilities in muscle AChE levels, brain AChE specific activities remained grossly similar in all of these transgenic lines. These ranged from 160 ± 5.0 (S.E.M.) and 170 ± 15 (S.E.M.) nmol/min/mg for the mice carrying InE6-AChE and control FVB/N mice and reached 240 ± 21 (S.E.M.) and 230 ± 25 (S.E.M.) nmol/min/mg for the two I4 AChE pedigrees with muscle activities 25- and 350-fold higher than control. HpE6-AChE transgenics revealed the highest values, reaching 300 nmol/min/mg levels in brain, 50% higher than the brain activity in control FVB/N mice [3]. Table I presents these comparative data for

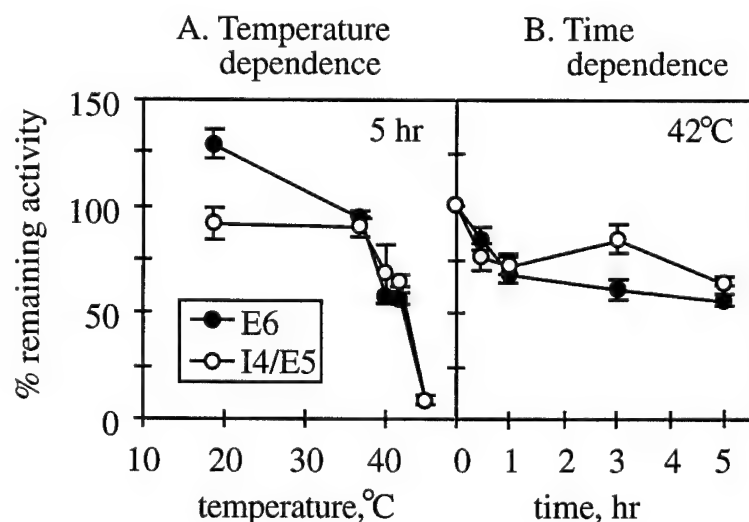
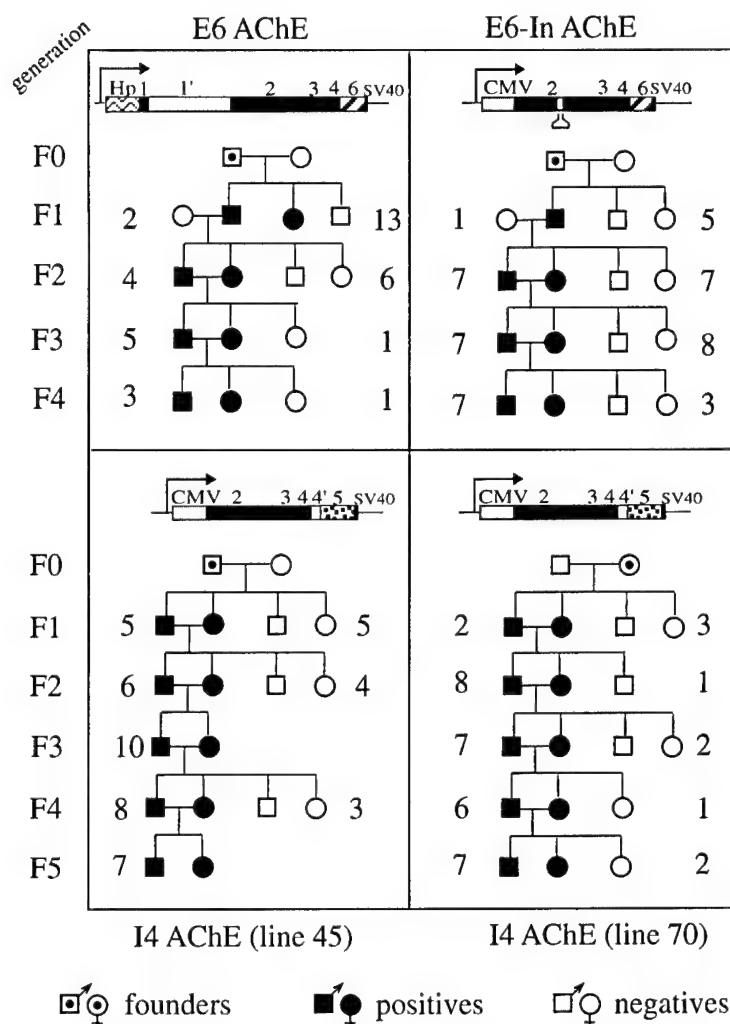


Figure 2. Heat stability of the recombinant AChE variants. Presented are relative ATCh hydrolysis rates of recombinant E6-AChE and I4-AChE variants. Enzymes were produced in microinjected *Xenopus* oocytes from the corresponding vectors including the CMV promoter (see figure 1). Twenty-four h post-injection, 1:10 w/v oocyte homogenates in high salt-detergent buffer [25] were incubated at various temperatures for 5 h (A) or at 42 °C for the noted time periods (B). Endogenous *Xenopus* AChE activity was measured in homogenates from non-injected oocytes and the corresponding values subtracted. Shown are average results of 2–3 experiments and standard errors of three measurements for each sample. Note that 'readthrough' I4 AChE of human origin is at least as stable as the synaptic E6-AChE variant.

Figure 3. Mendelian inheritance of transgenic AChE variants in mouse pedigrees. Presented are pedigree data for four distinct lines of mice carrying the noted transgenic AChE constructs. Transgene presence was determined by PCR amplification from tail DNA samples as previously detailed [3]. Numbers of positive and negative mice in each litter are noted on the left and right, respectively. Note the increase in positive litter mates within advanced generations, reflecting unimpaired Mendelian inheritance.



brain and muscle AChE levels, clearly demonstrating the distinct expression patterns of these transgenes in brain and muscle.

To test whether overexpression of AChE affects endogenous cholinesterase activity, butyrylcholinesterase (BChE) activity was determined in these homogenates by measurements of butyrylthiocholine hydrolysis in the presence and absence of the specific AChE inhibitor BW284C51 (table 1). These analyses showed an increase of 1.8 over control in muscle BChE activity in the I4-AChE transgenic line that showed a 350-fold increase of AChE activity. In contrast, decreased brain BChE activities to levels 0.46 and 0.58 of control were observed both in the InE6-AChE transgenic line and the I4-AChE transgenic line with the 25-fold increase in muscle AChE activity (line 45). The changes in BChE activities in both muscle and brain were thus much lower than those in AChE activities in these tissues.

4. Discussion

Creation of three novel transgenic mouse pedigrees revealed position effect variegations in muscle expression as well as brain-specific silencing of the CMV I4-AChE transgene encoding the secretory, soluble non-synaptic 'readthrough' form of AChE. When compared to transgenic mice carrying the InE6-AChE transgene and the corresponding catalytically active E6-AChE transgene, a pattern emerges which testifies to considerable permissivity of muscle AChE variations but maintains brain AChE levels within a very narrow activity window.

We found that two mouse pedigrees, both carrying apparently similar copy numbers of the I4 AChE transgene under control of the CMV promoter, express 25- and 350-fold excesses of AChE in muscle. This clearly reflects position effects of the insertion of these transgenes into the host mouse genome. In

Transgene PCR kinetics

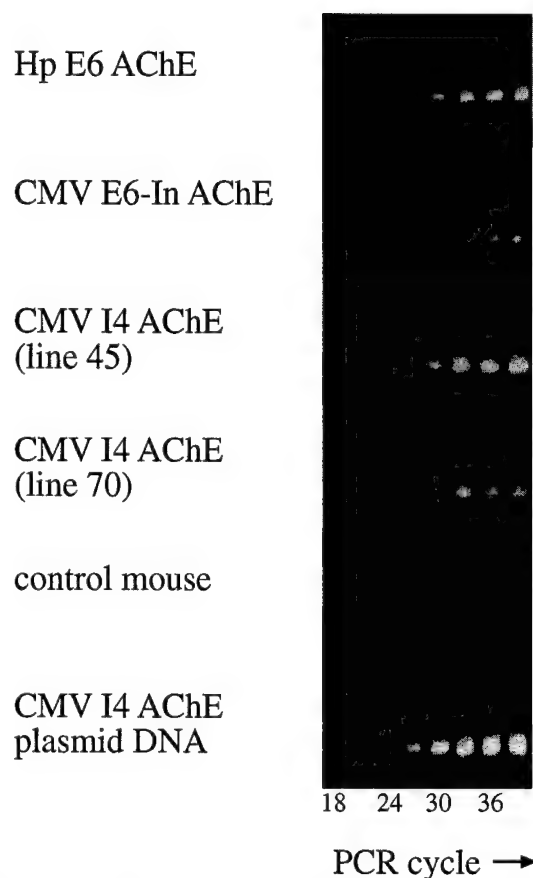


Figure 4. PCR indications for differential gene dosage. Shown are kinetic accumulations of PCR products drawn every third cycle, from 500 ng tail DNA of positive F4-F7 generation mice from each of the noted transgenic pedigrees. Genomic DNA from a control mouse was incubated with primers selective for the AChE coding sequence to exclude the possibility of false positives. CMV I4-AChE plasmid DNA (10 pg) was used with the same primers to calibrate copy numbers. Note the parallel kinetics, indicating similar gene dosage, for lines 45 and 70, both of which carry CMV I4-AChE transgenes. One out of three reproducible analyses is presented for each transgene. PCR cycles in which samples were taken are noted under the gel panels.

both *Xenopus* embryos and transfected mammalian cells, the CMV promoter was found to be 20-fold more potent than the endogenous 586 bp HpACHE promoter [5, 12]. This, in turn, suggests enhancement of the CMV promoter activity in muscle by neighboring sequences at the transgene integration site in at least one of the I4 AChE pedigrees. Moreover, in several other tissues of these transgenic

lines, a pattern of overexpression similar to that of muscle was observed (data not shown). Our I4 AChE pedigrees are therefore a good example of positive effects imposed by the chromosomal integration sites, unlike many other cases where the *cis*-acting elements included in transgenes were insufficient to overcome the negative effects imposed by the integration site. This phenomenon of repressed expression of genes translocated to abnormal integration sites was much earlier termed 'chromosomal positioning effect' [7, 18, 26]. The observation of enhanced I4-AChE production in our transgenic animals therefore suggests integration at sites favorable for transcription.

Selective activation or silencing of mammalian genes in specific cells and tissues is regulated by locus control regions that act in *cis* to ensure correct functioning of adjacent transcription units in all cells of a certain lineage [15]. This operates through the establishment of an open chromatin configuration and requires several domains within the locus control regions [2, 8]. The massive overexpression of muscle AChE in the analyzed transgenic mice may therefore reflect integration in the proximity of such region(s). That this overexpression apparently does not interfere with neuromuscular properties is compatible with the non-synaptic localization of I4 AChE as well as with its minimal effect on neuromuscular junction development [22, 25].

Within the same mouse pedigrees with massive muscle AChE increases, variations in brain AChE activity were remarkably limited. It is unlikely that this silencing was due to the known phenomenon of transgene repeats [9], as it was limited to brain and did not affect muscle expression. Rather, it is likely that the viability of these transgenic pedigrees depended on minimal changes in their brain AChE levels, so that the only lineages that survived were those where the transgene's expression in the brain was effectively silenced. This suggests that the upstream domains adjacent to the two I4 AChE transgenes should include effective brain silencing in addition to muscle enhancing elements and calls for testing their composition and the mechanisms of their function in comparison with other regulators [27].

The reduced brain AChE levels in the InE6-AChE transgenics suggest active expression of this transgene as well as competition of its protein product with host E6-AChE on its translation, post-translational processing and/or deposition sites. That muscle AChE activity was somewhat increased in these mice may further reflect feedback responses of those neuromuscular structures where integration of inactive AChE caused an artificial increase in ACh levels. These newly created transgenic models should therefore be most valuable for testing the biological

effects of AChE overproduction and sustained inhibition and for exploring the molecular mechanisms controlling both the expression patterns of these transgenes and the ability of the host mice to accommodate these changes.

Acknowledgments

This study has been supported by the U.S.-Israel Binational Science Foundation (Grant No. 96-00110 to H.S. and J.P.), and by grants to H.S. by the U.S. Army Medical Research and Development Command (Grant No. DAMD 17-97-1-7007), the Israel Ministry of Defense and Ester, Ltd., Neuroscience (Tel Aviv and Boston). All experiments were performed in accordance with Institutional guidelines for animal experimentation. We gratefully acknowledge the assistance of Dr. R. Beeri and Ms. T. Milai and R. Abdul Ghani with experiments.

References

- [1] Andres C., Beeri R., Friedman A., Lev-Lehman E., Henis S., Timberg R., Shani M., Soreq H., AChE transgenic mice display embryonic modulations in spinal cord CHAT and neurexin I β gene expression followed by late-onset neuro-motor deterioration, *Proc. Natl. Acad. Sci. USA* 94 (1997) 8173–8178.
- [2] Aronow B.J., Ebert C.A., Valerius M.T., Potter S.S., Wiginton D.A., Witte D.P., Hutton J.J., Dissecting a locus control region: facilitation of enhancer function by extended enhancer-flanking sequences, *Mol. Cell Biol.* 15 (1995) 1123–1135.
- [3] Beeri R., Andres C., Lev-Lehman E., Timberg R., Huberman T., Shani M., Soreq H., Transgenic expression of human acetylcholinesterase induces progressive cognitive deterioration in mice, *Curr. Biol.* 5 (1995) 1063–1071.
- [4] Beeri R., Leovere N., Mervis R., Huberman T., Grauer E., Changeux J.P., Soreq H., Enhanced hemicholinium binding and attenuated dendrite branching in cognitively impaired AChE-transgenic mice, *J. Neurochem.* 69 (1997) 2441–2451.
- [5] Ben Aziz-Aloya R., Seidman S., Timberg R., Sternfeld M., Zakut H., Soreq H., Expression of a human acetylcholinesterase promoter-reporter construct in developing neuromuscular junctions of *Xenopus* embryos, *Proc. Natl. Acad. Sci. USA* 90 (1993) 2471–2475.
- [6] Dobie K.W., Lee M., Fantes J.A., Graham E., Clark A.J., Springbett A., Lathe R., McClenaghan M., Variegated transgene expression in mouse mammary gland is determined by the transgene integration locus, *Proc. Natl. Acad. Sci. USA* 93 (1996) 6659–6664.
- [7] Dobzhansky T., Position effects on genes, *Biol. Rev.* 11 (1936) 364–434.
- [8] Festenstein R., Tolaini M., Corbella P., Mamalaki C., Partridge J., Fox M., Milliou A., Jones M., Kioussis D., Locus control region function and heterochromatin-induced position effect variegation, *Science* 271 (1996) 1123–1125.
- [9] Garrick D., Fiering S., Martin D.I.K., Whitelaw E., Repeat-induced gene silencing in mammals, *Nature Genet.* 18 (1998) 56–59.
- [10] Grosveld F., Kollias G., Transgenic animals, Academic Press, London, 1992.
- [11] Holms C., Jones S.A., Budd, T.C., Greenfield S.A., Non-cholinergic, trophic action of recombinant acetylcholinesterase on mid-brain dopaminergic neurons, *J. Neurosci. Res.* 49 (1997) 207–218.
- [12] Karpel R., Sternfeld M., Ginzberg D., Guhl E., Graessman A., Soreq H., Overexpression of alternative human acetylcholinesterase forms modulates process extensions in cultured glioma cells, *J. Neurochem.* 66 (1996) 114–123.
- [13] Kaufer D., Friedman A., Seidman S., Soreq H., Acute stress facilitates long-lasting changes in cholinergic gene expression, *Nature* 393 (1998) 373–377.
- [14] Kaufer D., Friedman A., Seidman S., Soreq H., Anticholinesterases induce multigenic transcriptional feedback response suppressing cholinergic neurotransmission, *Chem. Biol. Interact.* (1998), in press.
- [15] Kioussis D., Festenstein R., Locus control regions: overcoming heterochromatin-induced gene inactivation in mammals, *Curr. Opin. Genet. Dev.* 17 (1997) 614–618.
- [16] Knapp M.J., Knopman D.S., Solomon P.R., Pendlebury W.W., Davis C.S., Gracon S.I., A 30-week randomized controlled trial of high-dose tacrine in patients with Alzheimer's disease, *J. Am. Med. Assoc.* 271 (1994) 985–991.
- [17] Layer P.G., Willbold E., Novel functions of cholinesterases in development, physiology and disease, *Prog. Histochem. Cytochem.* 29 (1995) 1–99.
- [18] Lewis E.B., The phenomenon of position effect, *Adv. Genet.* 3 (1950) 73–115.
- [19] Palmiter R.D., Brinster R.L., Germline transformation of mice, *Annu. Rev. Genet.* 20 (1986) 465–495.
- [20] Sabi J.F., Henikoff S., Copy number and orientation determine the susceptibility of a gene to silencing by nearby heterochromatin in *Drosophila*, *Genetics* 142 (1996) 447–458.
- [21] Seidman S., Ben-Aziz Aloya R., Timberg R., Loewenstein Y., Velan B., Shafferman A., Liao J., Norgaard-Pedersen B., Brodbeck U., Soreq H., Overexpressed monomeric human acetylcholinesterase induces subtle ultrastructural modifications in developing neuromuscular junctions of *Xenopus laevis* embryos, *J. Neurochem.*, 62 (1994) 1670–1681.
- [22] Seidman S., Sternfeld M., Ben Aziz-Aloya R., Timberg R., Kaufer-Nachum D., Soreq H., Synaptic and epidermal accumulations of human acetylcholinesterase is encoded by alternative 3'-terminal exons, *Mol. Cell. Biol.* 15 (1995) 2993–3002.
- [23] Shapira M., Seidman S., Sternfeld M., Timberg R., Kaufer D., Patrick J.W., Soreq H., Transgenic engineering of neuromuscular junctions in *Xenopus laevis* embryos transiently overexpressing key cholinergic proteins, *Proc. Natl. Acad. Sci. USA*, 91 (1994) 9072–9076.
- [24] Small D.H., Reed, G., Whitefield B., Nurcombe V., Cholinergic regulation of neurite outgrowth from isolated chick sympathetic neurons in culture, *J. Neurosci.* 15 (1995) 144–151.
- [25] Sternfeld M., Ming G.-L., Song H.-J., Sela K., Poo M.-M., Soreq H., Acetylcholinesterase enhances neurite growth and synapse development through alternate contributions of its hydrolytic capacity, core protein and variable C-termini, *J. Neurosci.*, 18 (1998) 1240–1249.
- [26] Sturtevant A.H., The effect of unequal crossover at the Bar locus in *Drosophila*, *Genetics* 10 (1925) 117–147.
- [27] Sutherland H.G.E., Martin D.I.K., Whitelaw E., A globin enhancer acts by increasing the proportion of erythrocytes expressing a linked transgene, *Mol. Cell. Biol.* 17 (1997) 1607–1614.

Muscarinic acetylcholine receptors activate the acetylcholinesterase gene promoter

Roger M. Nitsch^a, Stefan Rossner^b, Claudia Albrecht^a, Manuel Mayhaus^a, Janna Enderich^a, Reinhard Schliebs^b, Michael Wegner^a, Thomas Arendt^b, H. von der Kammer^a

^aCenter for Molecular Neurobiology and Alzheimer's Disease Research Group, University of Hamburg, Martinistr. 52, 20246 Hamburg, Germany

^bDepartment of Neurochemistry, Paul-Flechsig-Institute for Brain Research, Jahnallee 59, 04109 Leipzig, Germany

Abstract – The acetylcholinesterase (AChE) gene promoter contains several overlapping binding sites for Sp1 and Egr-1 transcription factors. Cotransfection experiments and promoter assays showed that Egr-1 can potently activate transcription from the human AChE promoter. Muscarinic acetylcholine receptors (mAChR) rapidly activate, via protein kinase C-mediated signaling, expression of the *Egr-1* gene, leading to dramatically increased nuclear concentrations of Egr-1 protein, and to increased binding of Egr-1 to specific DNA recognition sequences. These mAChR-induced increases are followed by increased transcription from the human AChE promoter. In vivo studies with intraventricular infusions of the cholinergic immunotoxin 192 IgG saporin showed more than 80% decrease of AChE activity in cholinergic target areas of the hippocampus and brain cortex. The results are compatible with a combination of decreased AChE activity in degenerating subcortical cholinergic projections, and additional decreases in postsynaptic AChE gene expression. Together our data show that mAChR can activate transcription from the AChE promoter via increased synthesis of Egr-1. The results suggest a feedback mechanism by which the AChE gene is activated by cholinergic neurotransmission, possibly leading to increased formation of AChE protein and accelerated degradation of acetylcholine at cholinergic synapses. This possibility suggests testing of cholinomimetic compounds currently in development for the treatment of Alzheimer's disease for their potential ability to increase AChE gene expression. (©Elsevier, Paris)

Résumé — Les récepteurs muscariniques activent le promoteur de l'acétylcholinestérase. Le promoteur du gène de l'acétylcholinestérase (AChE) contient plusieurs sites chevauchant pour les facteurs de transcription Sp1 et Egr-1. Des expériences de co-transfection ont montré que le facteur Egr-1 active puissamment le promoteur de l'AChE humaine. Les récepteurs muscariniques (mAChR) induisent une expression rapide du gène *Egr-1*, par la voie de la protéine kinase C, augmentant considérablement le niveau de la protéine Egr-1 dans le noyau et sa liaison aux éléments de réponse sur l'ADN. L'activation des récepteurs muscariniques augmente ainsi la transcription contrôlée par le promoteur du gène humain d'AChE. L'injection intraventriculaire in vivo de l'immunotoxine cholinergique saporine-IgG192 provoque une diminution de plus de 80 % de l'activité de l'AChE dans les cibles cholinergiques de l'hippocampe et dans le cortex. Ces effets peuvent s'expliquer par une diminution de l'activité de l'AChE dans les projections cholinergiques subcorticales en dégénérescence, accompagnée d'une diminution de l'expression post-synaptique de l'expression d'AChE. L'ensemble de nos résultats montre que les récepteurs muscariniques peuvent activer la transcription du gène de l'AChE en stimulant la synthèse d'Egr-1. Ces données suggèrent un mécanisme de rétro-contrôle, dans lequel le gène de l'AChE est activé par la neurotransmission cholinergique, ce qui peut augmenter la production d'AChE et accélérer l'hydrolyse de l'acétylcholine dans les synapses cholinergiques. Il serait donc intéressant de voir si les composés cholinomimétiques actuellement en cours de développement pour le traitement de la maladie d'Alzheimer peuvent augmenter l'expression de l'AChE. (©Elsevier, Paris)

muscarinic acetylcholine receptors / acetylcholinesterase / Egr-1 / APP / amyloid / Alzheimer's disease / AChE inhibitors / muscarinic agonists

1. Introduction

Muscarinic acetylcholine receptors (mAChR) are a family of cell surface receptors with seven transmembrane domain topology. They couple to G protein-mediated regulation of a complex variety of intracellular signaling pathways that involve the phospholipases A₂, C, and D, protein kinase C (PKC), mitogen activated protein (MAP) kinase, Jun kinase (JNK), tyrosine kinases, adenylyl cyclase, intracellular Ca²⁺ levels, as well as the regulation of K⁺ and Ca²⁺ channels (for reviews, see [26, 81–83]). Among the many cellular responses induced by

mAChR is the regulation of posttranslational modification of two proteins with central roles in the pathophysiology of Alzheimer's disease: presenilin 1 (PS1) and β -amyloid precursor protein (APP). Stimulated mAChR increases the phosphorylation of the PS1 C-terminus, and it increases proteolytic α -secretase processing of APP within its β -amyloid (A β) domain, thus reducing the amount of A β peptides secreted into extracellular compartments [19, 32, 38, 57–61, 79]. In mammalian brain, muscarinic neurotransmission is involved in several aspects of neuronal plasticity including learning and memory [14, 21, 22, 56, 84]. Muscarinic transmission is im-

paired in several CNS disorders associated with memory loss, including AD [25] (for review see [40]), as well as in the memory decline associated with old age [20]. Together, these observations promoted the development of such cholinomimetic drugs as acetylcholinesterase (AChE) inhibitors and muscarinic agonists for the treatment of AD. Several AChE inhibitors and muscarinic agonists drugs are tested currently in clinical trials [5, 13, 36, 52, 72]. Some of these compounds received FDA-approval for the treatment of AD.

AChE is a serine hydrolase that terminates the action of acetylcholine (ACh) at cholinergic synapses by hydrolyzing it [65, 73] (for reviews, see [45, 46, 76]). The human AChE gene promoter contains consensus binding sites for Sp1, Egr-1 (*zif/268*, *Krox24*, *NGFI-A* [7, 39, 48]) and AP2 transcription factors, and deletion experiments demonstrated that the overlapping Sp1 and Egr-1 sites are essential for activation of AChE gene expression, whereas AP2 repressed it [24, 29]. Importantly, the activities of AChE gene promoters may be regulated differently in mouse and man. In contrast to the possible repression by Egr-1 of the murine AChE gene promoter [41, 53], the human AChE gene promoter is strongly activated by Egr-1, as indicated by cotransfection experiments with *Egr-1* expression constructs [78]. *Egr-1* is a member of the early growth response (*Egr*) gene family of nuclear transcription factors with immediate-early gene induction kinetics, and with zinc-finger DNA binding domain structure [74, 75]. *Egr-1* expression is rapidly inducible by a broad variety of external stimuli including transsynaptic neuronal excitation, differentiation cues, mitogens and tissue injury (for review, see [23]). In the central nervous system, electrical high-frequency stimulation of the perforant path, for example, induces *Egr-1* expression in postsynaptic target cells of the dentate gyrus [9]. This increase is blocked by NMDA agonists suggesting that it is mediated by glutamate via activation of NMDA receptors [7]. Moreover, serotonin, noradrenalin, dopamine, bradykinin, vasopressin, nicotine and muscarinic agonists can stimulate *Egr-1* expression in a variety of cell types suggesting that Egr-1 mediates long-term transsynaptic responses triggered by a broad variety of neurotransmitters and neuromodulators [1, 8, 10, 18, 30, 34, 50, 62, 69, 70, 78, 80, 85].

Because the AChE gene is a target for Egr-1-mediated transcriptional regulation, and because *Egr-1* expression can be stimulated by mAChR, we tested, by using cotransfection experiments, whether mAChR can regulate the AChE gene promoter via expression of *Egr-1*. In addition, we used the 192 IgG-saporin lesion model of cholinergic differentiation to test whether AChE expression in cholinergic

target areas is under the control of subcortical cholinergic projection neurons.

2. Materials and methods

Cell culture experiments, Northern blots, Western blots, mobility shift assays, cotransfections and promoter analyses were done as described [78].

2.1. Immunotoxin lesions

Cholinergic immunolesions were performed according to Rossner et al. [66–68]. Rats were anesthetized with 60 mg ketamine i.m. and 40 mg nembutal i.p./kg body weight, and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). The skull was opened at bregma, 0.8 mm and +1.2 mm lateral to the longitudinal suture by placing a small hole. Stereotaxic infusion of 4.0 µg 192 IgG-saporin (Chemicon Int., Temecula, CA, USA) in 10 µL PBS, pH 7.4, was performed at a depth of 3.4 mm below the cortical surface at a rate of 1.0 µL per minute into the left lateral ventricle using a Hamilton syringe with a 31 gage needle (Hamilton, Bonaduz, Switzerland). After injection the syringe was kept for an additional 5 min at the injection site to allow complete diffusion. Brains were analyzed 7 and 14 days after 192 IgG-saporin infusion. Vehicle controls were processed identically, but received PBS only. Untreated controls were used for normalizations of both 192 IgG-saporin-treated animals and vehicle controls. These experiments were approved by the Committee for Animal Experimentation at Regierungspräsidium Leipzig.

2.2. AChE histochemistry

Frozen tissue sections were processed for AChE histochemistry according to the protocol of Andrä and Lojda [2]. Briefly, the sections were preincubated with 0.1 M Tris-maleate buffer, pH 5.6, for 30 min. Slices were incubated in reaction medium that contained 0.4 M sodium citrate, 0.12 M cupric sulfate, 0.16 M potassium ferricyanide, and 50 mg acetylthiocholine iodide in 0.1 M Tris-maleate buffer in the presence of the cholinesterase inhibitor tetraisopropylpyrophosphoramide (10 µM, Sigma, St. Louis, USA). Incubations were done at 37 °C for 30 min. Sections from the individual treatment groups were processed in parallel, and they were treated identically. Sections were digitized with a CCD camera, and reaction intensities were quantitated by densitometry.

3. Results

The results of these studies clearly showed that stimulation of mAChR with the muscarinic agonist carbachol rapidly increased transcription of the *Egr*-family of transcription factors Egr-1, Egr-2, Egr-3 and Egr-4 [78]. Western blots showed that nuclear levels of Egr-1 protein were undetectable in the basal, unstimulated conditions, and that mAChR stimulation dramatically increased nuclear levels of Egr-1 protein. We also demonstrated, by using mo-

bility shift assays, that Egr-1 synthesized in response to m1AChR stimulation bound to an Egr-specific DNA recognition sequence, and that this interaction was blocked by anti-Egr-1 antibodies. Pharmacological experiments indicated that the m1AChR-induced mobility shift of the Egr recognition site was blocked by atropine, strongly supporting a specific, mAChR-mediated effect of carbachol. Similar to m1AChR activation, stimulation of PKC with the phorbol ester phorbol myristate acetate (PMA) led to increased Egr-1 binding in the mobility shift assay, and down-regulation of PKC by chronic PMA treatment reduced it. Together, these results indicated a role of PKC in the coupling of Egr-1-mediated transcriptional regulation to m1AChR activity. We next demonstrated, by co-transfection, that carbachol can increase transcription from a minimal Egr-1-responsive promoter fused to a luciferase reporter construct. Additional co-transfection experiments with the human AChE gene promoter fused to a luciferase reporter and a CMV-driven expression plasmid for *Egr-1* demonstrated that Egr-1 dramatically increased transcription from the AChE gene promoter. Co-expression of the human AChE gene promoter construct with m1AChR demonstrated that carbachol increased transcription from the promoter. Again, this increase was blocked by atropine indicating that m1AChR can increase the expression of the AChE gene, and the increase was mimicked by PMA, but not by 8-bromo-cAMP [78].

In order to test the relevance of these observations in vivo, we reduced the cholinergic input from the subcortical projection system into the target areas in brain cortex and hippocampus by infusing the cholinergic immunotoxin 192 IgG-saporin into the lateral ventricles of adult rats. 192 IgG-saporin dramatically reduced histochemical staining of AChE activity in cortex and hippocampus within 7 and 14 days. In addition, AChE activity dropped significantly, after 14 days, in the cholinergic cell bodies in the medial septum (figure 2). Densitometric analyses showed that magnitudes of these decreases were in the range of 80% as compared both to the untreated control condition and to the injected vehicle controls that underwent identical stereotaxic infusion procedures (figure 3).

4. Discussion

Our study showed that muscarinic m1AChR can activate the promoter of the AChE gene. Internal signaling pathways that coupled mAChR to the AChE gene promoter included protein kinase C and increased transcription of the *Egr-1* gene, leading to increased cellular concentrations of *Egr-1* mRNA

within 10 min of stimulation. This increase is followed by more than 100-fold increases in nuclear concentrations of Egr-1 protein within 2 h of m1AChR stimulation, and the increase was blocked by atropine, supporting a specific mAChR-mediated mechanism. Egr-1 proteins synthesized in response to m1AChR stimulation bound to Egr-specific DNA recognition sequences as evidenced by electrophoretic mobility shift assays, and antibodies against EGR-1 blocked this interaction, demonstrating its specificity. Promoter assays showed that stimulation of m1AChR increased transcription either from a minimal Egr-1-responsive promoter, or from the AChE gene promoter. In addition, expression of Egr-1 readily increased transcription of the AChE gene promoter. Increased transcription from both promoters was blocked by atropine and it was mimicked by PMA, but not by 8-bromo-cAMP, suggesting a role for PKC, but not PKA, in the coupling of the AChE gene promoter to mAChR. If confirmed in brain, these data suggest a feedback mechanism in the regulation of muscarinic neurotransmission by which cells stimulated with ACh increase the formation of AChE that, in turn accelerates the termination of ACh action at cholinergic synapses (figure 1).

In order to test whether muscarinic regulation of AChE gene expression is relevant in vivo, we reduced the cholinergic input into the cortex and the hippocampus of rats by 192 IgG-saporin immunolesion. Presynaptic terminals of the basal forebrain cholinergic projection neurons selectively take up this immunotoxin, and it kills these cells. Other brain cells, including striatal cholinergic interneurons, for example, remain unaffected by this treatment. The

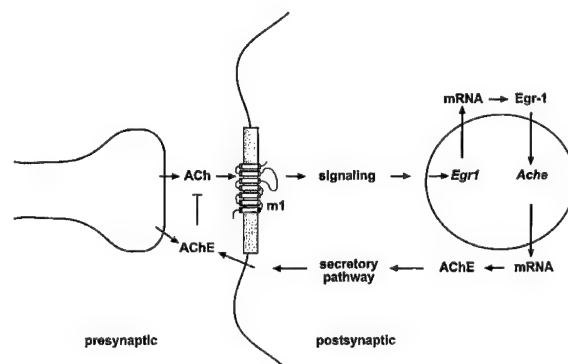


Figure 1. Proposed mechanism of the cholinergic control of AChE expression. We have shown that stimulated m1AChR increases *Egr-1* mRNA and Egr-1 protein that binds to, and activates Egr-1 responsive promoters including the AChE gene promoter. The proposed increase in AChE mRNA, protein and secretion is hypothetical, but supported by independent recent observations [35] (A. Nordberg, personal communication; see *Discussion*).

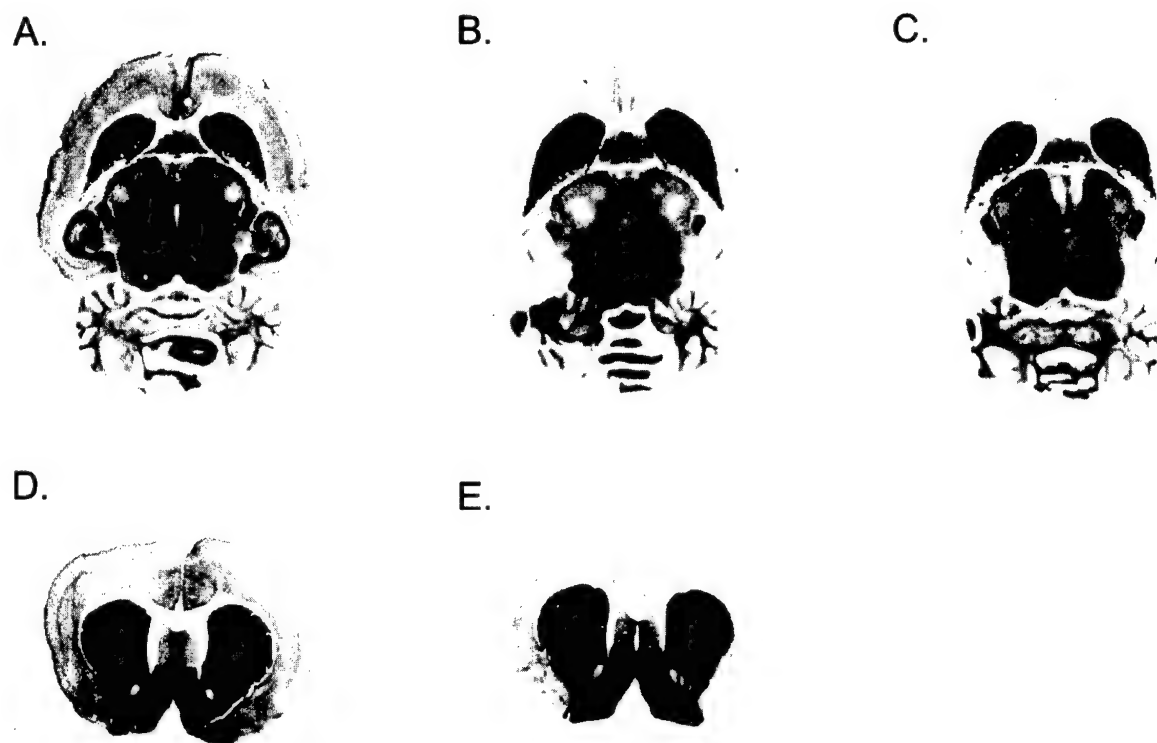


Figure 2. 192 IgG-saporin reduced AChE activity in cholinergic target areas. 192 IgG-saporin or vehicle (**A, D**) was infused into the lateral ventricles of adult rats and AChE histochemistry was done 7 days (**B, E**) or 14 days (**C**) following the infusion. 192 IgG-saporin reduced AChE activity in the hippocampus and throughout the cerebral cortex within 7 days, and AChE activity also decreased in the medial septum 14 days after infusion (**C**). In contrast, AChE activity in the cholinergic interneurons of the striatum and the thalamus remained unaffected by the immunotoxin.

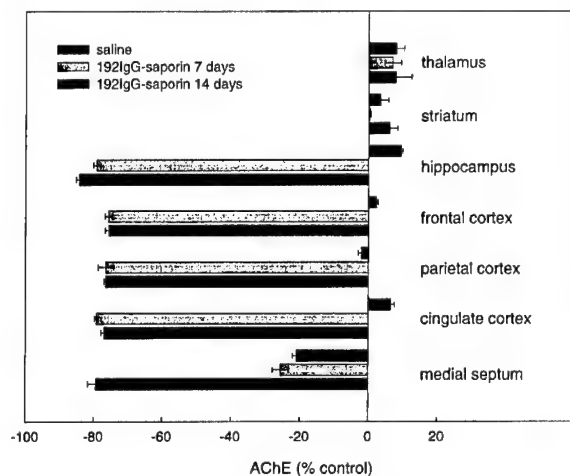


Figure 3. Densitometric analyses of AChE reductions induced by 192 IgG-saporin. Densitometry was done in the brain regions indicated, and they were normalized to untreated normal controls. In comparison to the vehicle controls, 192 IgG-saporin decreased AChE activity in hippocampus, frontal cortex and cingulate cortex by more than 80%.

192 IgG-saporin immunolesion causes cognitive deficits in the Morris water maze [42] consistent with the known role of the subcortical cholinergic projection neurons for learning and memory in rats [21, 84]. In our experiments, 192 IgG-saporin caused a dramatic reduction in AChE activity throughout the brain cortex and the hippocampus within 7 days, and low AChE activities were maintained for at least 14 days (*figure 2*). AChE in the medial septum decreased within 14 days of the immunolesion, but AChE remained unchanged both in the thalamus and in the striatum (*figure 2C*). Densitometric analyses showed that the magnitudes of these decreases were in the order of 80 to 90% as compared to either the vehicle controls of the untreated controls (*figure 3*).

These data are consistent with the concept that AChE gene expression in postsynaptic cholinergic target cells is under the control of presynaptic cholinergic input. In the mammalian brain, the AChE gene is normally expressed by pyramidal neurons throughout the cortex, in addition to high levels of expression in the striatum and the basal forebrain

[6, 11, 27, 28]. Therefore, AChE activities in cholinergic target areas are derived from two sources: first from axonal transport by the subcortical cholinergic projection neurons, and second, from endogenous synthesis of AChE in cortical and hippocampal neurons. It is thus likely that the decrease in AChE activity caused by the 192 IgG-saporin lesion reflects the sum of decreases associated with degenerating presynaptic cholinergic afferences and decreased activity-dependent AChE gene expression in cortical and hippocampal cholinergic target cells (see *figure 1*).

The concept of cholinergic regulation of AChE gene expression is supported by three additional independent observations: First, the AChE inhibitor pyridostigmine dramatically increased AChE gene expression in hippocampal brain slices *in vitro* [35]. These data strongly suggest that higher tissue levels of ACh in hippocampal cholinergic target regions can increase AChE gene expression in hippocampal cells. Second, clinical observations showed that treatments of AD patients with the cholinesterase inhibitor tacrine for 12 months significantly increased AChE activity in CSF by 50%, as compared to baseline. In contrast, AChE activities in CSF of untreated matched AD patients remained stable over a time period of 17 months (Agneta Nordberg, personal communication). Third, early studies from the mid eighties demonstrated that the systemic application of the AChE inhibitor physostigmine increased AChE activities in CSF in an atropine-sensitive manner, suggesting the involvement of muscarinic receptors [47]. Together, these findings underscore the concept that the regulation of AChE gene expression in brain is activity-dependent, and that it is under the control of external signals including muscarinic receptors. Muscarinic receptor subtype analyses showed that, in addition to m1AChR, the m2, m3, and m4AChR can also stimulate the transcription of the *egr-1* gene, although to different extents [78]. It is therefore possible that AChE gene expression in presynaptic cholinergic neurons is also under the control of ACh produced and secreted by the same neurons.

In addition to Egr-mediated signaling, mAChR are known to increase the expression of the immediate early genes *c-jun*, *jun D*, *c-fos*, *fos B*, *fra-1* and *nur-77* [3, 4, 12, 16, 31, 34, 77]. Because these transcription factors regulate the expression of a broad variety of target genes, it is possible that muscarinic receptor activity is involved in the activity-dependent regulation of these target genes as well, possibly contributing to activity-dependent plastic responses of postsynaptic cells. Known target genes of Egr-1 regulation include the growth factors PDGF, β FGF, and TGF- β 1 [23, 44, 71, 80], the prohormone con-

vertase 2 [33] as well as the interleukin-2 receptor beta-chain [43], suggesting roles in mitogenic responses and in the regulation of cell growth and differentiation. An additional example for an Egr-1-dependent target is the phenylethanolamine *N*-methyltransferase gene in the adrenal gland [49]. It encodes the enzyme that converts norepinephrine to epinephrine, and the expression of this gene is known to be under the control of muscarinic stimulation [17, 50, 51]. In addition, Egr-1 can be involved in apoptosis, either activating it via a p53-mediated cell death pathway, or suppressing it, depending on the cell biological context [54, 55].

4.1. Possible clinical implications

AD is associated with decreased cholinergic neurotransmission due to the early degeneration and loss of the long cholinergic afferences to the cerebral cortex and hippocampus [40, 86]. This deafferentation is associated with decreased AChE activities in brain cortex and hippocampus [15, 63, 64]. In the cerebral cortex of AD patients, AChE activity is significantly reduced in pyramidal neurons, despite unchanged densities of neurons visualized by Nissl stains of adjacent sections [28]. Our observation of muscarinic regulation of AChE gene expression suggests that this loss of AChE in cortical pyramidal cells could reflect decreased muscarinic stimulation of AChE gene expression, rather than loss of AChE positive neurons.

The activity-dependent expression of Egr-1 mediates important neuronal functions related to stimulus-induced plasticity, cell growth, long-term potentiation and synaptic reorganization. These functions may be impaired in post-synaptic neurons as a result of the cholinergic deafferentation. Because many of these functions are regulated by the activity-dependent expression of Egr-1-dependent target genes that may be also under cholinergic control, it is possible that expression of these genes in AD brain is decreased as well. Postmortem studies are required to test this hypothesis.

It is well established that, besides the degeneration of subcortical cholinergic projection neurons in AD, postsynaptic mAChR expressed by cholinergic target cells are preserved in AD cortex and hippocampus. Thus, cholinomimetic compounds were designed to replace the ACh deficit, and to stimulate postsynaptic cholinergic target neurons. As a result, several AChE inhibitors as well as muscarinic agonists are currently under development and tested in clinical trials for the treatment of AD. Consistently, the AChE inhibitors appear to reduce the rate of progression of clinical and neuropsychological measures of dementia severity, but the magnitudes of these ef-

fects are limited, and dementia continues to progress, after an initial halt, within several months of treatment. If confirmed in human brain, limited efficacy of these compounds may be related to the possibility that they increase AChE gene expression via Egr-1-mediated transcriptional activation. Thus, in vivo studies are required to test whether pharmacological treatments designed to stimulate mAChRs are associated with increased AChE gene expression, increased AChE protein levels, and increased enzyme activity with accelerated breakdown of acetylcholine. If so, additional pharmacological concepts need to be developed that aim at maintaining the beneficial effects of mAChR stimulation without the possible unwanted side effect of increased AChE gene transcription.

Acknowledgments

Supported by Deutsche Forschungsgemeinschaft Ni482/2, Bundesministerium für Bildung und Forschung und Americal Health Organization (Alzheimer Forschung Initiative e.V.).

References

- [1] Abu-Shakra S.R., Cole A.J., Drachman D.B., Nerve stimulation and denervation induce differential patterns of immediate early gene mRNA expression in skeletal muscle, *Mol. Brain Res.* 18 (1993) 216–220.
- [2] Andrä J., Lojda Z., A histochemical method for the demonstration of acetylcholinesterase activity using semipermeable membranes, *Histochemistry* 84 (1986) 575–579.
- [3] Altin J.G., Kujubu D.A., Raffioni S., Eveleth D.D., Herschmann H.R., Bradshaw R.A., Differential Induction of primary-response (TIS) genes in PC12 pheochromocytoma cells and the unresponsive variant PC12nnr5, *J. Biol. Chem.* 266 (1991) 5401–5406.
- [4] Bernard V., Dumartin B., Lamy E., Bloch B., Fos immunoreactivity after stimulation or inhibition of muscarinic receptors indicates anatomical specificity for cholinergic control of striatal efferent neurons and cortical neurons in the rat, *Eur. J. Neurosci.* 5 (1993) 1218–1225.
- [5] Bodick N.C., Offen W.W., Levey A.I., Cutler N.R., Gauthier S.G., Satlin A., Shannon H.E., Tollefson G.D., Rasmussen K., Bymaster F.P., Hurley D.J., Potter W.Z., Paul S.M., Effects of xanomeline, a selective muscarinic receptor agonist, on cognitive function and behavioral symptoms in Alzheimer disease, *Arch. Neurol.* 54 (1997) 465–473.
- [6] Carson KA, Geula C, Mesulam MM (1991) Electron microscopic localization of cholinesterase activity in Alzheimer's brain tissue, *Brain Res* 540, 204–208.
- [7] Christy B.A., Lau L.F., Nathans D., A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with 'zinc finger' sequences, *Proc. Natl. Acad. Sci. USA* 85 (1988) 7857–7861.
- [8] Cole A.J., Abu-Shakra A., Saffen D.W., Baraban J.M., Worley P.F., Rapid rise in transcription factor mRNAs in rat brain after electroshock-induced seizures, *J. Neurochem.* 55 (1990) 1920–1927.
- [9] Cole A.J., Saffen D.W., Baraban J.M., Worley P.F., Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation, *Nature* 340 (1989) 474–476.
- [10] Cole A.J., Bhat R.V., Patt C., Worley P.F., Baraban J.M., D1 dopamine receptor activation of multiple transcription factor genes in rat striatum, *J. Neurochem.* 58 (1992) 1420–1426.
- [11] Coleman A.E., Geula C., Price B.H., Mesulam M.M., Differential laminar distribution of acetylcholinesterase and butyrylcholinesterase containing tangles in the cerebral cortex of Alzheimer's disease, *Brain Res.* 595 (1992) 340–344.
- [12] Coso O.A., Chiariello M., Kalinec G., Kyriakis J.M., Woodgett J., Gutkind J.S., Transforming G protein-coupled receptors potently activate JNK (SAPK), *J. Biol. Chem.* 270 (1995) 5620–5624.
- [13] Cummings J.L., Cyrus P.A., Bieber F., Mas J., Orazem J., Gulanski B., Metrifonate treatment of the cognitive deficits of Alzheimer's disease. Metrifonate Study Group, *Neurology* 50 (1998) 1214–1221.
- [14] Damasio A.R., Graff-Radford N.R., Eslinger P.J., Damasio H., Kassel N., Amnesia following basal forebrain lesions, *Arch. Neurol.* 42 (1985) 263–271.
- [15] Davies P., Neurotransmitter-related enzymes in senile dementia of the Alzheimer type, *Brain Res.* 171 (1979) 319–227.
- [16] Dragunow M., Abraham W., Hughes P., Activation of NMDA and muscarinic receptors induces nur-77 mRNA in hippocampal neurons, *Mol. Brain Res.* 36 (1996) 349–356.
- [17] Ebert S.N., Wong D.L., Differential activation of the rat gene by Sp1 and Egr-1, *J. Biol. Chem.* 270 (1995) 17299–17305.
- [18] Ebihara T., Saffen D., Muscarinic acetylcholine receptor-mediated induction of zif268 mRNA in PC12D cells requires protein kinase C and the influx of extracellular calcium, *J. Neurochem.* 68 (1997) 1001–1010.
- [19] Farber S.A., Nitsch R.M., Schulz J.G., Wurtman R.J., Regulated secretion of β -amyloid precursor protein in rat brain, *J. Neurosci.* 15 (1995) 7442–7451.
- [20] Fischer W., Chen K.S., Gage F.H., Bjorklund A., Progressive decline in spatial learning and integrity of forebrain cholinergic neurons in rats during aging, *Neurobiol. Aging* 13 (1992) 9–23.
- [21] Fischer W., Sirevaag A., Wiegand S.J., Lindsay R.M., Bjorklund A., Reversal of spatial memory impairments in aged rats by nerve growth factor and neurotrophins 3 and 4/5 but not by brain-derived neurotrophic factor, *Proc. Natl. Acad. Sci. USA* 91 (1994) 8607–8611.
- [22] Furey M.L., Pietrini P., Haxby J.V., Alexander G.E., Lee H.C., VanMeter J., Grady C.L., Shetty U., Rapoport S.I., Schapiro M.B., Freo U., Cholinergic stimulation alters performance and task-specific regional cerebral blood flow during working memory, *Proc. Natl. Acad. Sci. USA* 94 (1997) 6512–6516.
- [23] Gashler A., Sukhatme V.P., Early growth response protein 1 (Egr-1): Prototype of a zinc-finger family of transcription factors, *Progr. Nucleic Acid. Res.* 50 (1995) 191–224.
- [24] Getman D.K., Mutero A., Inoue K., Taylor P., Transcription factor repression and activation of the human acetylcholinesterase gene, *J. Biol. Chem.* 270 (1995) 23511–23519.
- [25] Geula G., Mesulam M.M., Systematic regional variations in the loss of cortical cholinergic fibers in Alzheimer's disease, *Cereb. Cortex* 6 (1996) 165–177.

- [26] Gutkind J.S., The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades, *J. Biol. Chem.* 27 (1998) 1839–1842.
- [27] Hammond P., Rao R., Koenigsberger C., Brimiljoin A., Regional variation in expression of acetylcholinesterase mRNA in adult rat brain analyzed by *in situ* hybridization, *Proc. Natl. Acad. Sci. USA* 91 (1994) 10933–10937.
- [28] Heckers S., Geula C., Mesulam M.M., Acetylcholinesterase-rich pyramidal neurons in Alzheimer's disease, *Neurobiol. Aging* 13 (1992) 455–460.
- [29] Huang R.P., Fan Y., Ni Z., Mercola D., Adamson E.D., Reciprocal modulation between Sp1 and Egr-1, *J. Cell. Biochem.* 66 (1997) 489–499.
- [30] Hughes P., Dragunow M., Activation of pirenzepine-sensitive muscarinic receptors induces a specific pattern of immediate-early gene expression in rat brain neurons, *Mol. Brain Res.* 24 (1994) 166–178.
- [31] Hughes P., Dragunow M., Muscarinic receptor-mediated induction of Fos protein in rat brain, *Neurosci. Lett.* 150 (1992) 122–126.
- [32] Hung A.Y., Haass C., Nitsch R.M., Qiu W.Q., Citron M., Wurtman R.J., Growdon J.H., Selkoe D.J., Activation of protein kinase C inhibits cellular production of the amyloid β -protein, *J. Biol. Chem.* 268 (1993) 22959–22962.
- [33] Jansen E., Ayoubi T.A., Meulemans S.M., van De Ven W.J., Regulation of human prohormone convertase 2 promoter activity by the transcription factor EGR-1, *Biochemistry* 328 (1997) 69–74.
- [34] Katayama N., Iwata E., Sakurai H., Tsuchiya T., Tsuda M., Additive induction of Egr-1 (*zif/268*) mRNA expression in neuroblastoma \times glioma hybrid NG108-15 cells via cholinergic muscarinic, α_2 -adrenergic, and bradykinin receptors, *J. Neurochem.* 60 (1993) 902–907.
- [35] Kaufer D., Friedmann A., Seidman S., Soreq H., Acute stress facilitates long-lasting changes in cholinergic gene expression, *Nature* 393 (1998) 373–377.
- [36] Knapp M.J., Knopman D.S., Solomon P.R., Pendlebury W.W., Davis C.S., Gracon S.I., A 30-week randomized controlled trial of high-dose tacrine in patients with Alzheimer's disease. The Tacrine Study Group, *J. Am. Med. Assoc.* 271 (1994) 985–991.
- [37] Kornhauser J.M., Nelson D.E., Mayo K.E., Takahashi J.S., Regulation of jun-B messenger RNA and AP-1 activity by light and a circadian clock, *Science* 255 (1992) 1581–1584.
- [38] Langer U., Albrecht C., Mayhaus M., Velden J., Wiegmann H., Klaudiny J., Müller D., von der Kammer H., Nitsch R.M., in: Younkin S.G., Tanzi R.E., Christen Y. (Eds.), *Presenilins and Alzheimer's Disease*, Springer Berlin, Heidelberg, New York, 1998, pp. 79–84.
- [39] Lemaire P., Revelant O., Bravo R., Charnay P., Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells, *Proc. Natl. Acad. Sci. USA* 85 (1988) 4691–4695.
- [40] Levey A.I., Muscarinic acetylcholine receptor expression in memory circuits: implications of treatment of Alzheimer's disease, *Proc. Natl. Acad. Sci. USA* 93 (1996) 12541–12546.
- [41] Li Y., Camp S., Rachinsky T.L., Bongiorno C., Taylor P., Promoter elements and transcriptional control of the mouse acetylcholinesterase gene, *J. Biol. Chem.* 268 (1993) 3563–3572.
- [42] Lin L., Leblanc C.J., Deacon T.W., Isaacs O., Chronic cognitive deficits and amyloid precursor protein elevation after selective immunotoxin lesions of the basal forebrain cholinergic system, *Neuroreport* 9 (1998) 547–552.
- [43] Lin J.X., Leonnard W.J., The immediate-early gene product Egr-1 regulates the human interleukin-2 receptor beta-chain promoter through noncanonical Egr and Sp1 binding sites, *Mol. Cell. Biol.* 17 (1997) 3714–3722.
- [44] Liu C., Adamson E., Mercola D., Transcription factor EGR-1 suppresses the growth and transformation of human HT-1080 fibrosarcoma cells by induction of transforming growth factor beta 1, *Proc. Natl. Acad. Sci. USA* 93 (1996) 11831–11836.
- [45] Massoulié J., Pezzementi L., Bon S., Krejci E., Vallette F.M., Molecular and cellular biology of the cholinesterases, *Progr. Neurobiol.* 41 (1993) 31–91.
- [46] Massoulié J., Legay C., Anselmet A., Krejci E., Coussen F., Bon S., Biosynthesis and integration of acetylcholinesterase in the cholinergic synapse, *Progr. Brain Res.* 109 (1996) 55–65.
- [47] Mattio T., McIlhenny E., Giacobini E., Hallak M., The effects of physostigmine on acetylcholinesterase activity of CSF, plasma and brain. A comparison of intravenous and intraventricular administration in beagle dogs, *Neuropharmacology* 25 (1985) 1167–1177.
- [48] Milbrand J., A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor, *Science* 238 (1987) 797–799.
- [49] Morita K., Ebert S.N., Wong D.L., Role of transcription factor Egr-1 in Phorbol Ester-induced phenylethanolamine N-methyltransferase gene expression, *J. Biol. Chem.* 270 (1995) 11161–11167.
- [50] Morita K., Wong D.L., Role of Egr-1 in cholinergic stimulation of phenylethanolamine N-methyltransferase promoter, *J. Neurochem.* 67 (1996) 1244–1251.
- [51] Monta K., Bell R.A., Siddall B.J., Wong D.L., Neural stimulation of Egr-1 messenger RNA expression to rat adrenal gland: Possible relation to phenylethanolamine N-methyltransferase gene regulation, *J. Pharmacol. Exp. Therap.* 279 (1996) 379–385.
- [52] Morris J.C., Cyrus P.A., Orazem J., Mas J., Bieber F., Ruzicka B.B., Gulanski B., Metrifonate benefits cognitive, behavioral, and global function in patients with Alzheimer's disease, *Neurology* 50 (1998) 1222–1230.
- [53] Mutero A., Camp S., Taylor P., Promoter elements of the mouse acetylcholinesterase gene, *J. Biol. Chem.* 270 (1995) 1822–1872.
- [54] Muthukumar S., Han S.S., Rangnekar V.M., Bondada S., Role of Egr-1 gene expression in B-cell receptor-induced apoptosis in an immature B cell lymphoma, *J. Biol. Chem.* 271 (1997) 27987–27993.
- [55] Nair P., Muthukumar S., Sells S.F., Han S.-S., Sukhatme V.P., Rangnekar V.M., Early growth response-1-dependent apoptosis is mediated by p53, *J. Biol. Chem.* 272 (1997) 20131–20138.
- [56] Nilsson O.G., Leanza G., Rosenblad C., Lappi D.A., Wiley R.G., Bjorklund A., Spatial learning impairments in rats with selective immunolesion of the forebrain cholinergic system, *Neuroreport* 3 (1992) 1005–1008.
- [57] Nitsch R.M., Slack B.E., Wurtman R.J., Growdon J.H., Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors, *Science* 258 (1992) 304–307.
- [58] Nitsch R.M., Farber S.A., Growdon J.H., Wurtman R.J., Release of amyloid β -protein precursor derivatives by electrical

- depolarization of rat hippocampal slices, *Proc. Natl. Acad. Sci. USA* 90 (1993) 5191–5193.
- [59] Nitsch R.M., Growdon J.H., Role neurotransmission in the regulation of amyloid β -protein processing, *Biochem. Pharmacol.* 47 (1994) 1275–1284.
- [60] Nitsch R.M., From acetylcholine to amyloid: Neurotransmitters and the pathology of Alzheimer's disease, *Neurodegen.* 5 (1996) 477–482.
- [61] Nitsch R.M., Deng M., Growdon J.H., Wurtman R.J., Serotonin 5-HT_{2a} and 5-HT_{2c} receptors stimulate amyloid precursor protein ectodomain secretion, *J. Biol. Chem.* 271 (1996) 4188–4914.
- [62] O'Donovan K.J., Wilkens E.P., Baraban J.M., Sequential expression of Egr-1 and Egr-3 in hippocampal granule cells following electroconvulsive stimulation, *J. Neurochem.* 70 (1998) 1241–1248.
- [63] Perry E.K., Johnson M., Kerwin J.M., Piggot M.A., Court J.A., Shaw P.J., Ince P.G., Brown A., Perry R.H., Convergent cholinergic activities in aging and Alzheimer's disease, *Neurobiol. Aging* 13 (1992) 393–400.
- [64] Perry E.K., Perry R.H., Blessed G., Tomlinson B.E., Changes in brain cholinesterases in senile dementia of Alzheimer type, *Neuropathol. Appl. Neurobiol.* 4 (1978) 273–277.
- [65] Rachinsky T.L., Camp S., Li Y., Ekström T.J., Newton M., Taylor P., Molecular cloning of mouse acetylcholinesterase: tissue distribution of alternatively spliced mRNA species, *Neuron* 5 (1990) 317–327.
- [66] Rossner S., Perez-Polo J.R., Schliebs W.R., Bigl V., Differential expression of immediate early genes in distinct layers of rat cerebral cortex after selective immunolesion of the forebrain cholinergic system, *J. Neurosci. Res.* 38 (1994) 282–293.
- [67] Rossner S., Schliebs W.R., Perez-Polo J.R., Bigl V., Differential changes in cholinergic markers from selected brain regions after specific immunolesion of rat cholinergic basal forebrain system, *J. Neurosci. Res.* 40 (1995) 31–43.
- [68] Rossner S., Schliebs W.R., Härtig W., Bigl V., 192IgG-saporin-induced immunotoxic lesion of cholinergic basal forebrain system: neurochemical effects on cholinergic neurotransmission in rat cerebral cortex and hippocampus, *Brain Res. Bull.* 38 (1995) 371–381.
- [69] Rupprecht H.D., Dann P., Sukhatme V.P., Sterzel R.B., Coleman D.L., Effect vasoactive agents on induction of Egr-1 in rat mesangial cells: correlation with mitogenicity, *Am. J. Physiol.* 263 (1992) F623–F636.
- [70] Rupprecht H.D., Sukhatme V.P., Lacy J., Sterzel R.B., Coleman D.L., PDGF-induced Egr-1 expression in rat mesangial cells is mediated through upstream serum response elements, *Am. J. Physiol.* 265 (1993) F351–F360.
- [71] Silverman E.S., Khachigian L.M., Lindner V., Williams A.J., Collins T., Inducible PDGF A-chain transcription in smooth muscle cells is mediated by Egr-1 displacement of Sp1 and Sp3, *Am. J. Physiol.* 273 (1997) H1415–H1426.
- [72] Solomon P.R., Knapp M.J., Gracon S.I., Groccia M., Pendlebury W.W., Long-term tacrine treatment in patients with Alzheimer's disease, *Lancet* 348 (1996) 275–276.
- [73] Sternfeld M., Ming G., Song H., Sela K., Timberg R., Poo M., Soreq H., Acetylcholinesterase enhances neurite growth and synapse development through alternative contributions of its hydrolytic capacity, core protein, and variable termini, *J. Neurosci.* 18 (1998) 1240–1249.
- [74] Sukhatme V.P., Cao X.M., Chang L.C., Tsai-Morris C.H., Stamenkovich D., Ferreira P.C., Cohen D.R., Edwards S.A., Shows T.B., Curran T., Le Beau M.M., Adamson E.D., A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization, *Cell* 53 (1988) 37–43.
- [75] Swirnoff A.H., Milbrandt J., DNA-binding specificity of NGFI-A and related zinc-finger transcription factors, *Mol. Cell. Biol.* 15 (1995) 2275–2287.
- [76] Taylor P., Radic Z., The cholinesterases: from genes to proteins, *Annu. Rev. Pharmacol. Toxicol.* 34 (1994) 281–320.
- [77] Trejo J.A., Brown J.H., c-fos and c-jun are induced by muscarinic receptor activation of protein kinase c but are differentially regulated by intracellular calcium, *J. Biol. Chem.* 266 (1990) 7876–7882.
- [78] von der Kammer H., Mayhaus M., Albrecht C., Enderich J., Wegner M., Nitsch R.M., Muscarinic acetylcholine receptors activate expression of the Egr gene family of transcription factors, *J. Biol. Chem.* 273 (1998) 14538–14544.
- [79] Walter J., Grünberg J., Capell A., Pesold B., Schindzielorz A., Citron M., Mendla K., George-Hyslop P.S., Multhaup G., Selkoe D.J., Haass C., Proteolytic processing of the Alzheimer disease-associated presenilin-1 generates an in vivo substrate for protein kinase C, *Proc. Natl. Acad. Sci. USA* 94 (1997) 5349–5354.
- [80] Wang D., Mayo M.W., Baldwin A.S. Jr., Basic fibroblast growth factor transcriptional autoregulation requires EGR-1, *Oncogene* 17 (1997) 2291–2299.
- [81] Wess J., Molecular basis of muscarinic acetylcholine receptor function, *TIPS* 14 (1993) 308–314.
- [82] Wess J., Molecular basis of muscarinic acetylcholine receptor function, *Trends Pharmacol. Sci.* 14 (1993) 308–313.
- [83] Wess J., G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition, *FASEB J.* 11 (1997) 346–354.
- [84] Winkler J., Suhr S.T., Gage F.H., Thal L.J., Fisher L.J., Essential role of neocortical acetylcholine in spatial memory, *Nature* 375 (1995) 484–487.
- [85] Worley P.F., Christy B.A., Nakabeppu Y., Bhat R.V., Cole A.J., Baraban J.M., Constitutive expression of zif268 in neocortex is regulated by synaptic activity, *Proc. Natl. Acad. Sci. USA* 88 (1991) 5106–5110.
- [86] Arendt T., Brückner M.K., Bigl V., Marcova L., Dendritic reorganization in the basal forebrain under degenerative conditions and its defects in Alzheimer's disease. II. Aging, Yarsakoff's disease, Parkinson's disease and Alzheimer's disease, *J. Comp. Neurol.* 351 (1995) 189–222.

Structure-function analysis of muscarinic acetylcholine receptors

E. Kostenis, F.-Y. Zeng, J. Wess*

Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Building 8A, Room B1A-05, Bethesda, Maryland 20892, USA

Abstract — The structural basis underlying the G protein coupling selectivity of different muscarinic receptor subtypes was analyzed by using a combined molecular genetic/biochemical approach. These studies led to the identification of key residues on the receptors as well as the associated G proteins that are critically involved in determining proper receptor/G protein recognition. Mutational analysis of the m3 muscarinic receptor showed that most native cysteine residues are not required for productive receptor/G protein coupling. The putative extracellular disulfide bond was found to be essential for efficient trafficking of the receptor protein to the cell surface but not for receptor-mediated G protein activation. (©Elsevier, Paris)

Résumé — Analyse structure-fonction des récepteurs muscariniques à l'acétylcholine. La base structurale sous-jacente des différents sous-types de récepteurs muscariniques, liés sélectivement aux protéines G, est analysée par une approche combinée de génétique moléculaire et de biochimie. Ces études ont mené à l'identification de résidus clefs sur les récepteurs ainsi que sur les protéines G associées impliquées de façon critique dans la reconnaissance appropriée (ou particulière) récepteur/protéine G. (©Elsevier, Paris)

muscarinic receptors / mutagenesis studies / receptor-G protein coupling / receptor structure / cysteine mutagenesis

1. Molecular basis of G protein coupling selectivity

The five muscarinic receptors (m1–m5) are prototypical members of the superfamily of G protein-coupled receptors (GPCRs). Based on their differential G protein-coupling properties, these receptors can be classed into two major functional categories (for a review, see Wess [21]): the odd-numbered muscarinic receptors, m1, m3, and m5, preferentially couple to G proteins of the G_q/G_{11} family, resulting in the hydrolysis of phosphoinositide lipids mediated by activation of different phospholipase C β isoforms. The m2 and m4 receptors, on the other hand, are primarily linked to G proteins of the G_i/G_o class, which, at a biochemical level, mediate the inhibition of adenylyl cyclase.

1.1. Functionally critical residues on the receptor protein

Recently, mutational analysis of different muscarinic receptors has led to the identification of a series of single amino acids that are critical for proper G protein recognition [21]. These residues are predicted to be located within the second (i2) and third (i3) intracellular loops. Different molecular genetic approaches, including insertion [7] and random mutagenesis strategies [12], indicate that a limited num-

ber of primarily hydrophobic residues at the N-terminus of the i3 loop play a key role in determining receptor/G protein coupling selectivity. These residues are predicted to form a surface that maps to the hydrophobic side of an amphiphilic α -helix [1, 7, 20].

Analogously, systematic mutational analysis of the C-terminal portion of the i3 loop of different muscarinic receptors has identified a set of four mostly hydrophobic amino acids (corresponding to Ala488, Ala489, Leu492, and Ser493 in the rat m3 muscarinic receptor; *figure 1*) that also play a significant role in dictating coupling selectivity [6, 8, 14]. Since these residues are thought to be located in a region predicted to be α -helically arranged [1, 20], they define a second hydrophobic surface that is critical for G protein recognition (*figure 1*).

In addition to residues at the i3 loop/membrane junctions, amino acids in the i2 loop have also been implicated in determining receptor/G protein coupling selectivity. For example, studies with hybrid m2/m3 muscarinic receptors have identified four hydrophilic m3 muscarinic receptor residues (Ser168, Arg171, Arg176, and Arg183; *figure 1*) that are critical for selective recognition of G_q/G_{11} proteins [6]. Since little is known about the conformation of the i2 loop, the spatial arrangement of these amino acids remains to be elucidated.

According to the molecular model of the transmembrane receptor core proposed by Baldwin [2, 3], the two functionally critical receptor surfaces present at the N- and C-terminus of the i3 loop are predicted

* Correspondence and reprints.

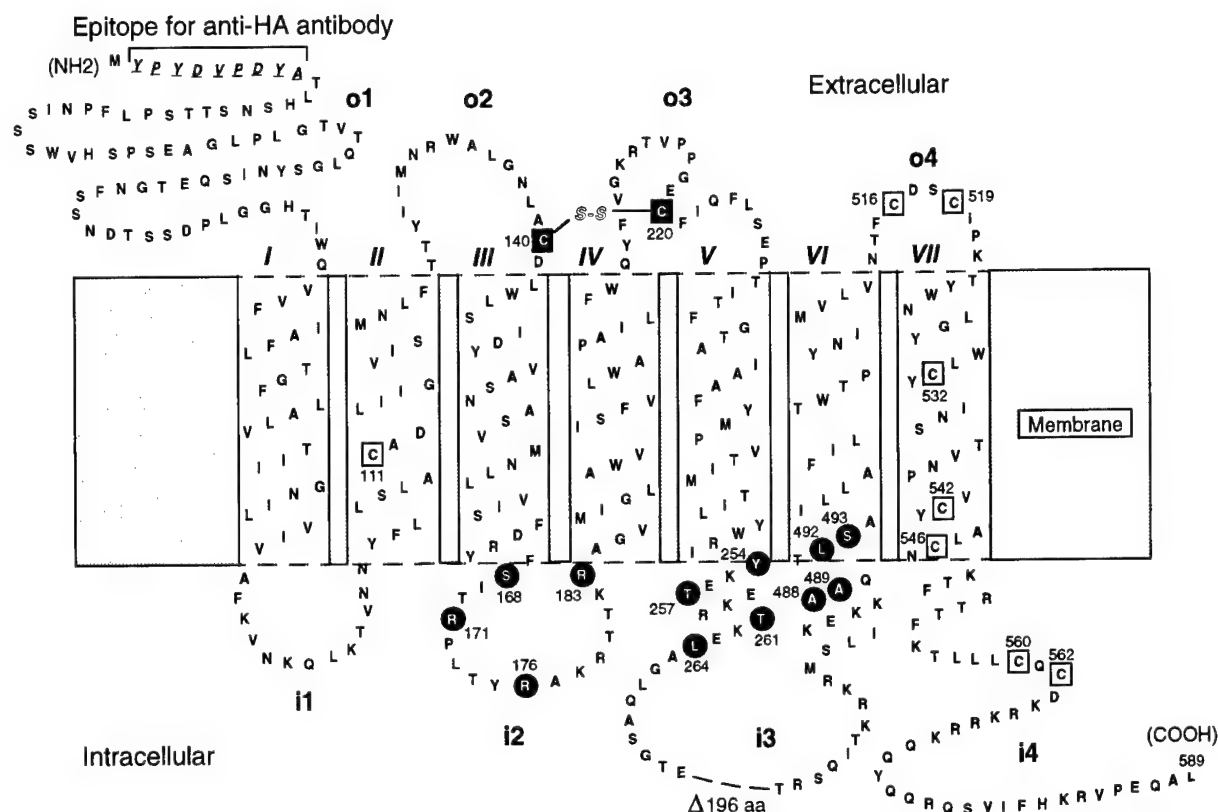


Figure 1. Transmembrane topology of the rat m3 muscarinic receptor. An N-terminal hemagglutinin (HA) epitope tag was introduced into mutant receptor constructs that were studied via immunological techniques (ELISA, confocal microscopy, etc.). Amino acids highlighted by filled circles have been shown to determine the G protein coupling selectivity of the m3 muscarinic receptor [21, 22]. Cysteine residues are boxed. The two extracellular cysteine residues that are conserved among most GPCRs and are predicted to be linked via a disulfide bridge are highlighted in black. Most of the i3 loop (196 amino acids), except for the N- and C-terminal 22 amino acids, can be deleted without affecting m3 receptor function (F.-Y. Zeng and J. Wess, unpublished results). Numbers refer to amino acid positions in the rat m3 muscarinic receptor sequence [4].

to project into the interior of the helical bundle, where they may be engaged in tertiary interactions with residues in adjacent receptor domains [22]. Since site-directed spin-labeling studies suggest that receptor activation is accompanied by an 'outward' movement of TM III and TM VI [10, 11], the resulting 'opening' of the intracellular receptor surface may allow G proteins to access the functionally important residues at the i3 loop/membrane junctions.

1.2. Functionally critical residues on the G protein α subunits

To better understand the molecular mechanisms governing receptor/G protein interactions, it is also important to identify specific regions/amino acids on the G protein(s) that are in contact with the receptor protein. A region that has been implicated most con-

sistently in receptor/G protein coupling is the extreme C-terminus of the G protein α subunit ($G\alpha$) [5, 19]. Consistent with this notion, we recently showed that the receptor selectivity of $G\alpha$ subunits can be changed by single C-terminal point mutations.

We initially employed a gain-of-function mutagenesis strategy to identify individual amino acids within the C-terminal tail of $\alpha_{i/o}$ subunits that are critical for receptor coupling selectivity [15]. Coexpression (in COS-7 cells) of a series of C-terminally modified mutant α_q subunits with the G_i -coupled m2 muscarinic receptor led to the identification of two mutant α_q subunits containing single $\alpha_q \rightarrow \alpha_i$ point mutations at their C-terminus which, in contrast to wild type α_q , were able to productively interact with the m2 receptor. This study provided the first example that the receptor coupling selectivity of G protein α subunits can be altered by single amino

acid substitutions [15]. The two single α_i residues present in the two mutant α_q constructs capable of efficiently interacting with the m2 muscarinic receptor were a -4 cysteine and a -3 glycine, respectively. These two residues are conserved among all members of the α_i/α_o protein family (with the exception of α_z which lacks the -4 cysteine).

In a subsequent study, Kostenis et al. [17] used a similar approach to map functionally important C-terminal α_q residues. In this case, amino acids at the C-terminus of α_s were replaced with the corresponding α_q residues, and the resulting mutant G proteins were studied (in cotransfected COS-7 cells) for their ability to gain coupling to the $G_{q/11}$ -linked m3 muscarinic receptor. This analysis led to the identification of two $\alpha_s \rightarrow \alpha_q$ single point mutants which gained the ability to functionally interact with the m3 muscarinic and other $G_{q/11}$ -coupled receptors [17]. The single α_q residues present in these constructs were a -5 glutamate and a -3 asparagine, respectively, which are found in all members of the $\alpha_{q/11}$ protein family.

Although these studies demonstrate that the C-terminus of $G\alpha$ is critically involved in receptor/G protein coupling, other regions of $G\alpha$ may also contribute to receptor binding and the selectivity of receptor/G protein interactions. Specifically, several lines of evidence suggest that the N-terminal portion of $G\alpha$ may be in contact with the receptor protein [5, 19].

Functional studies with an N-terminally truncated α_q subunit indicated that the N-terminal six amino acid extension characteristic for α_q and α_{11} subunits (MTLESI(M)) is critical for constraining the receptor coupling selectivity of these proteins [16]. When this sequence was removed by deletion mutagenesis, the resulting mutant α_q subunit gained the ability to be activated by the m2 muscarinic as well as other $G_{i/o}$ -coupled receptors which normally do not couple efficiently to wild type (full-length) α_q .

To study which specific amino acids within the N-terminal segment of $\alpha_{q/11}$ are critical for constraining the receptor coupling selectivity of these subunits, this region of α_q was subjected to systematic deletion and alanine scanning mutagenesis [18]. Surprisingly, coexpression studies in COS-7 cells showed that all 14 mutant G proteins studied (but not wild type α_q) gained the ability to productively interact with the $G_{i/o}$ -linked m2 muscarinic receptor. Similar results were obtained when we examined the ability of selected mutant α_q subunits to couple to the $G_{i/o}$ -coupled D2 dopamine and the G_s -coupled β_2 -adrenergic receptors. Additional experiments indicated that the functional promiscuity displayed by all investigated mutant α_q constructs was not due to overexpression (as compared with wild type α_q) or

initiation of translation at a downstream ATG codon (codon seven) [18]. These findings are consistent with the notion that the six-amino acid extension characteristic for $\alpha_{q/11}$ subunits forms a tightly folded protein subdomain that is critical for regulating the receptor coupling selectivity of these subunits. One possibility is that this subdomain has a gate function by selectively preventing access of $G_{i/o}$ - and G_s -coupled receptors. It is also conceivable that the six-amino acid extension exerts indirect conformational effects on the structure of $G\alpha_{q/11}$ that are crucial for maintaining the receptor selectivity of these subunits.

Interestingly, while all mutant α_q subunits incorporated significant amounts of [3H]palmitate, we noted that the strength of the palmitoylation signal was generally weaker, by about 40–75%, in the case of the mutant α_q subunits as compared to wild type α_q [18]. This observation raises the possibility that palmitoylation may play a role in regulating receptor/G protein coupling selectivity.

2. Cysteine mutagenesis as an approach to study muscarinic receptor structure and function

To elucidate the molecular mechanisms involved in muscarinic receptor activation, it would be highly desirable to obtain more direct structural information about the conformational differences between the resting and the activated receptor state. Currently, however, no high-resolution structural data are available for any GPCR, primarily due to difficulties in studying membrane proteins by X-ray crystallography or NMR.

To gain insight into muscarinic receptor structure, we are currently in the process of creating mutant receptors lacking native cysteine residues. Single cysteine residues will then be introduced into defined positions of the Cys-free mutant receptors, followed by their covalent modification by SH-group specific fluorescence agents, spin markers, or radio-labeled compounds with differing physicochemical properties. Such studies will allow the determination of the localization of specific amino acids with respect to the membrane/cytoplasm interface, the relative orientation of transmembrane helices, and the identity and orientation of secondary structure of targeted receptor regions.

We recently created three mutant m3 muscarinic receptors which contained no, two (Cys140 and Cys220), or three (Cys140, Cys220, and Cys532) remaining cysteine residues (resulting in m3 (C-less), m3 (C2), and m3 (C3), respectively) (figure 1). Surprisingly, both the m3 (C2) and m3 (C3) mutant receptors still retained the ability to stimulate

carbachol-dependent inositol phosphate production to the same maximum extent as the wild type receptor (F.-Y. Zeng, A. Soldner, and J. Wess, unpublished results), whereas the completely Cys-free receptor (m3 (C-less)) was functionally inactive. This observation indicated that the majority of native cysteine residues are not critical for m3 muscarinic receptor function.

Cys140 and Cys220 are highly conserved among most GPCRs of the rhodopsin family and are predicted to be linked via a disulfide bond [9, 13]. Interestingly, replacement of these residues by alanine in the m3 muscarinic receptor showed that this putative disulfide bond is not essential for protein stability (as determined via immunoblotting) and receptor-mediated G protein activation (studied in second messenger assays) (F.-Y. Zeng and J. Wess, unpublished results). However, ELISA and immunofluorescence studies demonstrated that the presence of both conserved cysteine residues is required for efficient trafficking of the m3 muscarinic receptor to the cell surface.

Notably, a mutant m3 receptor in which Cys140 and Cys220 were the only remaining cysteine residues was able to react with SH group-specific cross-linking agents as well as [¹⁴C]N-ethylmaleimide (F.-Y. Zeng and J. Wess, unpublished results), suggesting that disulfide bond formation between Cys140 and Cys220 may not be quantitative. These results shed new light on the functional role of the two extracellular cysteine residues present in most GPCRs.

References

- [1] Altenbach C., Yang K., Farrens D.L., Farahbakhsh Z.T., Khorana H.G., Hubbell W.L., Structural features and light-dependent changes in the cytoplasmic interhelical E-F loop region of rhodopsin: a site-directed spin labeling study, *Biochemistry* 35 (1996) 12470–12478.
- [2] Baldwin J.M., The probable arrangement of the helices in G protein-coupled receptors, *EMBO J.* 12 (1993) 1693–1703.
- [3] Baldwin J.M., Schertler G.F.X., Unger V.M., An alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein-coupled receptors, *J. Mol. Biol.* 272 (1997) 144–164.
- [4] Bonner T.I., Buckley N.J., Young A.C., Brann M.R., Identification of a family of muscarinic acetylcholine receptor genes, *Science* 237 (1987) 527–532.
- [5] Bourne H.R., How receptors talk to trimeric G proteins, *Curr. Opin. Cell Biol.* 9 (1997) 134–142.
- [6] Blin N., Yun J., Wess J., Mapping of single amino acid residues required for selective activation of G_{q/11} by the m3 muscarinic acetylcholine receptor, *J. Biol. Chem.* 270 (1995) 17741–17748.
- [7] Blüml K., Mutschler E., Wess J., Insertion mutagenesis as a tool to predict the secondary structure of a muscarinic receptor domain determining specificity of G-protein coupling, *Proc. Natl. Acad. Sci. USA* 91 (1994) 7980–7984.
- [8] Burstein E.S., Spalding T.A., Hill-Eubanks D., Brann M.R., Structure-function of muscarinic receptor coupling to G proteins: random saturation mutagenesis identifies a critical determinant of receptor affinity for G proteins, *J. Biol. Chem.* 270 (1995) 3141–3146.
- [9] Curtis C.A.M., Wheatley M., Bansal S., Birdsall N.J.M., Eveleigh P., Pedder E.K., Poyner D., Hulme E.C., Propylbenzylcholine mustard labels an acidic residue in transmembrane helix 3 of the muscarinic receptor, *J. Biol. Chem.* 264 (1989) 489–495.
- [10] Farahbakhsh Z.T., Ridge K.D., Khorana H.G., Hubbell W.L., Mapping light-dependent structural changes in the cytoplasmic loop connecting helices C and D in rhodopsin: a site-directed spin labeling study, *Biochemistry* 34 (1995) 8812–8819.
- [11] Farrens D.L., Altenbach C., Yang K., Hubbell W.L., Khorana H.G., Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin, *Science* 274 (1996) 768–770.
- [12] Hill-Eubanks D., Burstein E.S., Spalding T.A., Bräuner-Osborne H., Brann M.R., Structure of a G protein-coupling domain of a muscarinic receptor predicted by random saturation mutagenesis, *J. Biol. Chem.* 271 (1996) 3058–3065.
- [13] Kurtenbach E., Curtis C.A.M., Pedder E.K., Aitken A., Harris A.C.M., Hulme E.C., Muscarinic acetylcholine receptors: peptide sequencing identifies residues involved in antagonist binding and disulfide bond formation, *J. Biol. Chem.* 265 (1990) 13702–13708.
- [14] Liu J., Conklin B.R., Blin N., Yun J., Wess J., Identification of a receptor/G-protein contact site critical for signalling specificity and G-protein activation, *Proc. Natl. Acad. Sci. USA* 92 (1995) 11642–11646.
- [15] Kostenis E., Conklin B.R., Wess J., Molecular basis of receptor/G protein coupling selectivity studied by coexpression of wild type and mutant m2 muscarinic receptors with mutant G_{αq} subunits, *Biochemistry* 36 (1997) 1487–1495.
- [16] Kostenis E., Degtyarev M.Y., Conklin B.R., Wess J., The N-terminal extension of G_{αq} is critical for constraining the selectivity of receptor coupling, *J. Biol. Chem.* 272 (1997) 19107–19110.
- [17] Kostenis E., Gomez J., Lerche C., Wess J., Genetic analysis of receptor-G_{αq} coupling selectivity, *J. Biol. Chem.* 272 (1997) 23675–23681.
- [18] Kostenis E., Zeng F.-Y., Wess J., Functional characterization of a series of mutant G protein α_q subunits displaying promiscuous receptor coupling properties, *J. Biol. Chem.* (1998), in press.
- [19] Rens-Domiano S., Hamm H.E., Structural and functional relationships of heterotrimeric G-proteins, *FASEB J.* 9 (1995) 1059–1066.
- [20] Strader C.D., Fong T.M., Tota M.R., Underwood D., Dixon R.A.F., Structure and function of G protein-coupled receptors, *Annu. Rev. Biochem.* 63 (1994) 101–132.
- [21] Wess J., Molecular biology of muscarinic acetylcholine receptors, *Crit. Rev. Neurobiol.* 10 (1996) 69–99.
- [22] Wess J., G protein-coupled receptors: Molecular mechanisms involved in receptor activation and selectivity of G protein recognition, *FASEB J.* 11 (1997) 346–354.

Scanning mutagenesis of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor

Edward C. Hulme, Zhi-Liang Lu

Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK

Abstract — Scanning mutagenesis of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor has revealed a highly-differentiated α -helical structure. Lipid-facing residues are distinguished from a patch of residues which selectively stabilise the ground state of the receptor, and from a band of amino acids extending the full length of the helix, which contribute to the active agonist-receptor-G protein complex. The most important residues are strongly conserved in the GPCR superfamily. (©Elsevier, Paris)

Résumé — Mutagenèse du domaine transmembranaire 3 du récepteur à l'ACh muscarinique M₁. La mutagenèse montre que le TM3 possède une structure en hélice α comportant une bande d'AA correspondant à la zone de liaison avec les protéines G. (©Elsevier, Paris)

muscarinic / acetylcholine / G protein / mutagenesis / transmembrane

1. Introduction

Even when the structure of a protein is known at atomic resolution, site-directed mutagenesis studies are needed to understand the contribution of individual amino acids to its function. In the case of the G protein-coupled receptors (GPCRs), structural models are based on the analysis of sequence variations, in the context of a 6–8 Å resolution projection map of rhodopsin, determined by cryo-electron microscopy [1, 2]. Thus for the GPCRs, site-directed mutagenesis and chemical modification provide essential experimental information to refine the models, and to begin to understand receptor structure-function relationships.

Scanning mutagenesis, in which the side-chain interactions made by each amino acid in a given sequence are simplified, or eliminated, as a result of their sequential replacement by a smaller amino acid, commonly alanine [14], has provided a useful tool for the location of functional epitopes within receptors, and the analysis of their function [5]. Here we outline the application of this approach to transmembrane domain 3 (TMD 3) of the M1 muscarinic acetylcholine receptor (M1 mAChR). We show how the functional characterisation of the mutant proteins predicts the structure of the target sequence, gives information about its orientation within the three-dimensional structure of the receptor, and suggests possible functions for individual amino acids.

2. Materials and methods

Mutations were made by a PCR method as previously described [10]. Mutant receptors were expressed in COS-7 cells

under the control of an SV40 promoter. Ligand binding assays to measure the affinity of the antagonists N-[³H]-methyl-scopolamine (NMS), [³H]-quinuclidinyl benzilate (QNB) and agonists such as acetylcholine (ACh) were performed as described [10]. Assays of receptor function were performed by measuring ACh-stimulated phosphoinositide (PI) turnover [10]. Results were analysed by means of the extended ternary complex model of receptor function, as outlined below.

3. Results and discussion

3.1. The extended ternary complex model as a tool for the analysis of mutagenesis data

Mutation of amino acids in the M1 mAChR leads to a range of effects. One of the most striking is an increase in agonist affinity correlated with an enhancement of the basal functional activity. This phenotype is consistent with the operation of a two-state mechanism of agonist action, in which there is a pre-existing equilibrium, defined by an equilibrium constant K , between the active (R^*) and inactive (R) states of the receptor. Agonists bind with higher affinity to the R^* state than to the R state. A mutation which displaces the equilibrium in favour of the activated state, through an increase in the value of K , simultaneously increases both basal activity and agonist affinity.

The simplest receptor-response model to predict this behaviour is the extended ternary complex (EXTC) model of agonist action shown in *figure 1*. In this model, pre-existing (R^*) and agonist-induced (AR^*) complexes bind the G-protein, leading to the formation of catalytically active R^*G or AR^*G com-

plexes. These undergo GDP-GTP exchange, resulting in activation of the G protein. The EXTC model was first used to analyse the properties of a constitutively activated β -adrenergic receptor [12] and is widely applicable to GPCR mechanisms.

In using this model, we have assumed that the PI response, which is the functional response measured in our experiments, is directly proportional to the ratio of the concentration of activated-receptor G protein complex to total G protein, ie,

$$\begin{aligned} \text{PI/PI}_{\max} &= ([\text{AR}^*\text{G}] + [\text{R}^*\text{G}]) / [\text{GT}] \\ &= [\underline{\text{AR}^*\text{G}}] + [\underline{\text{R}^*\text{G}}] \end{aligned} \quad (1)$$

In equation 1, and the following equations, the underline is used to denote normalisation either by division by GT for the concentrations of complexes involving the receptor, or multiplication by GT in the case of the affinity constant KG.

For the wild-type M1 receptor expressed in COS-7 cells, measurement of the ACh dose-response curves after receptor blockade has shown that $\underline{\text{RT}}$ is about 20 for the wild-type receptor [10]. If $\underline{\text{RT}} \gg 1$, it can be shown that the solution to the EXTC model reduces to:

$$\begin{aligned} [\underline{\text{AR}^*\text{G}}] + [\underline{\text{R}^*\text{G}}] &= (\text{P2} + \text{P4}[\text{A}])[\underline{\text{RT}}] / \\ &= (1 + \text{P2}[\underline{\text{RT}}] + (\text{P4}[\underline{\text{RT}}] + \text{K}_{\text{Bin}})[\text{A}]) \end{aligned}$$

where $\text{P2} = \underline{\text{KKG}}$; $\text{P4} = \alpha \text{K}_\text{A} \underline{\text{KKG}}$, and $\text{K}_{\text{Bin}} = \text{K}_\text{A} (1 + \alpha \text{K})$.

This is an extension of equation 22 of Black and Leff [4].

There is usually no detectable GTP-shift in the agonist binding curves in membrane preparations from COS-7 cells, so K_{Bin} has been determined using the Hill equation:

$$\begin{aligned} ([\text{AR}] + [\text{AR}^*]) / [\text{RT}] &= \\ &= (\text{K}_{\text{Bin}}[\text{A}])^{n\text{H}} / (1 + (\text{K}_{\text{Bin}}[\text{A}])^{n\text{H}}) \end{aligned} \quad (2)$$

In general, dose-response data and binding data have been analysed by simultaneous fitting to equations (1) and (2).

By setting $[\text{A}] = 0$, we see that:

$$\text{Basal} = \text{P2}[\underline{\text{RT}}] / (1 + \text{P2}[\underline{\text{RT}}]) \quad (3)$$

while the apparent activation constant, K_{Act} , defined as $1/\text{EC}_{50}$, is:

$$\text{K}_{\text{Act}} = (\text{P4}[\underline{\text{RT}}] + \text{K}_{\text{Bin}}) / (1 + \text{P2}[\underline{\text{RT}}])$$

It follows that:

$$\text{P4} = (\text{K}_{\text{Act}} / (1 - \text{Basal}) - \text{K}_{\text{Bin}}) / [\underline{\text{RT}}] \quad (4)$$

Thus the parameters P2, P4 and K_{Bin} derived by fitting are well-determined by the measurable quantities evoked by the expression of the receptor in the cell, namely: i) the concentration of binding sites, $[\underline{\text{RT}}]$; ii) the basal activity; iii) the EC_{50} , derived from the PI assay; and iv) the binding constant, derived from an agonist binding experiment performed

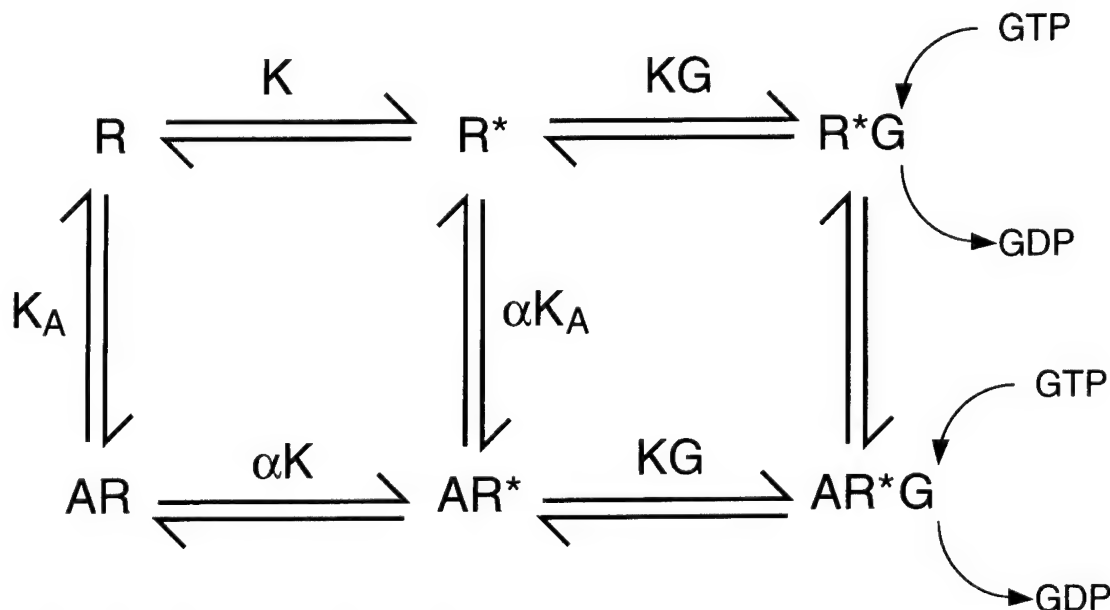


Figure 1. Extended ternary complex model of agonist-receptor-G protein interaction.

in parallel. Furthermore, it is possible, by combining them in various ways, to obtain information about the underlying molecular affinity constants of the model, thus:

$$P4/P2 = \alpha K_A \quad (5)$$

gives the affinity of the agonist for the R^*G complex and:

$$P4/K_{Bin} = \frac{KG\alpha K}{(1 + \alpha K)} \quad (6)$$

is a measure of the affinity of the G protein for the ensemble of $[AR]$ and $[AR^*]$. If an estimate of KG can be obtained, the values of αK for the various mutants can be calculated, and then used to estimate the value of K_A from the equation:

$$K_A = K_{Bin}/(1 + \alpha K) \quad (7)$$

Thus, under favourable circumstances and subject to certain explicit assumptions, the effects of mutations on the free energy associated with the ground-state binding of agonists (and antagonists), measured by K_A , can be separated from effects on the free energy of the activated state(s) relative to the ground state, i.e., of R^* relative to R (measured by K), of AR^* relative to AR (measured by αK), and of AR^*G relative to AR (measured by αKKG).

3.2. Scanning mutagenesis of transmembrane helix 3 of the M1 mAChR

Models of GPCRs suggest that TMD 3 forms the nucleus of the seven-fold helical bundle thought to make up the transmembrane structure [1, 2]. In the M1 mAChR, TMD 3 is initiated by a disulfide-bonded Cys [9]. It contains the binding site aspartate [9, 13], and terminates in amino acids which are important in G protein recognition, and in stabilising the receptor's tertiary structure [8, 10]. In rhodopsin, a change in the packing relationships between TMD 3 and the surrounding TMDs accompanies G protein activation [6], but the roles of most of the amino acids of TMD 3 in these processes are unknown.

We have carried out scanning mutagenesis on all of the amino acids between Leu100 and Tyr124 of the M1 mAChR, replacing each residue by Ala (or by Gly if the parent sequence itself contained Ala). The PI dose-response curves, and ACh binding curves measured after expression of the mutant receptors in COS-7 cells were analysed by the use of the EXTC model of receptor activation.

Two of the mutants provided a key to analyse this data set. These were S120A, and L116A.

S120A was expressed at 81% of the wild-type level. Unlike the wild-type receptor it gave significant

basal activity, equivalent to 6.6% of the ACh-stimulated maximum signal. The basal signal was inhibited by atropine with an IC_{50} of 10 nM.

In contrast, L116A was expressed at 2% of the wild-type level, and at this level gave no detectable basal PI signal. However, the ACh-stimulated maximum signal was equivalent to 40% of the wild-type maximum.

Postulating that the L116A mutation might destabilise the receptor structure, we attempted to counteract this effect by culturing the cells in the presence of 10^{-6} M atropine. This increased the expression level 50-fold, to 100% of the wild-type level. At this level, the L116A mutant gave high basal activity, equivalent to 70% of the maximum signal.

The L116A and S120A mutations increased basal activity, and agonist affinity, and decreased the slope of the agonist binding curve. This suggested that their primary effect is to increase the isomerisation constant, K , between the ground and activated states of the receptor, without changing KG . By combining the increase in the value of K , calculated from the basal activity (eqn. 3), with the values of $P4/K_{Bin}$ (eqn. 6), we estimated a value of 14.1 for KG . This led to values of αK of 0.83, 1.66 and 46 for the wild-type, S120A and L116A variants.

From equation (5), the values of αK_A calculated for S120A and L116A were 1.0×10^9 M $^{-1}$ and 4.8×10^8 M $^{-1}$ respectively. These values agree well, and suggest that the binding constant K_A and the cooperativity α of ACh binding are very similar for the two mutants. A cooperativity of about 8000 is compatible with the difference in affinity of high efficacy muscarinic agonists for the G protein-coupled and uncoupled states of the mAChRs [3]. The calculations predict that the affinity of ACh for the G protein-coupled state of the receptor is about 10^9 M $^{-1}$.

Utilising a value of 8000 for α gives values of K of about 10^{-4} , 2×10^{-4} and 5×10^{-3} for the wild-type, S120A and L116A receptors. These suggest that only a small proportion of the receptor naturally exists in the activated state (0.01%, wild-type, 0.02% S120A, 0.5% L116A) but that the fraction becomes substantial in the presence of ACh (45% wild-type, 62% S120A, 98% L116A).

It is interesting that the apparent 50-fold increase in the isomerisation constant of the L116A mutant was accompanied by a 50-fold decrease in its expression level. One possible explanation is that the mutation decreases the stability of the ground-state of the receptor, but without affecting the stability of the activated state. This would be expected to diminish the probability of successful receptor folding within the cell, and simultaneously to increase the probability of isomerisation to the activated state of those molecules which did fold successfully.

The value of 14.1 for K_G has been applied to estimate (subject to certain assumptions) the changes in K_A , and in the product αKKG for all of the mutants studied.

Figure 2 shows a helical net representation of the effects of the mutations on three parameters characterising the receptor. In each case, the change is shown relative to the wild-type receptor. The three parameters are: i) the receptor expression level, which may give an indication of the effect of the mutation on the stability of the ground state structure; ii) the formation of the AR binary complex relative to R; and iii) the formation of the ARG ternary complex relative to AR. Left-pointing arrows indicate a decrease and right-pointing arrows an increase in the stability of the AR binary complex relative to R (filled arrows), or the ARG ternary complex relative to AR (open arrows), while the diameter of the circle representing each amino acid is equal to the Log of the ratio of wild-type expression to mutant expression plus one. Reductions in the expression ratio or the K_A of less than three-fold, or in $AR \cdot G$ of less than six-fold are taken to be insignificant.

This representation gives insight into the probable roles of the various residues. The nine null residues, by the above criterion, are segregated on one face of the helix. Six occur in the outer half, between L100 and A111, but only three in the inner half, between S112 and Y124. In a Cys-substitution mutagenesis study of TMD 3 of the D2 dopamine receptor [7], nine of the twelve residues found to be unreactive with a polar thiol reagent correspond to the null residues in the M1 mAChR, and, with the exception of F121, are predicted to face towards the lipid bilayer in Baldwin's original model [1]. The two mutagenesis studies thus confirm the prediction of the model.

In contrast, only one of the residues whose mutation strongly affected receptor expression, D105, is found in the outer half, but six are found in the inner half of the helix. This is consistent with closer packing around the inner (more intracellular) than the outer (more extracellular) part of the helix.

On the inward-facing surface of the outer part of the helix, facing away from the lipid bilayer towards the core of the receptor, there is a patch of residues, centred on D105 and Y106 whose mutation strongly affected the formation of the ACh-receptor binary complex. With the exception of S109A, these mutations also reduced the affinity of the antagonists NMS and QNB. A subset of the mutations, W101A, D105A, Y106A and S109A also strongly reduced the formation of the ternary complex. However, mutation of two residues on the periphery of this patch, L102 and N110 primarily reduced ACh binary com-

plex formation, but had little further effect on ternary complex formation.

These findings reinforce the primacy of D105, supported by Y106, in ACh binding, and signal transduction [11, 13, 15]. Notably, D105 is the only residue whose mutation completely abolished signalling. However, D105 appears to have a complex function. It may contribute to the stability of the

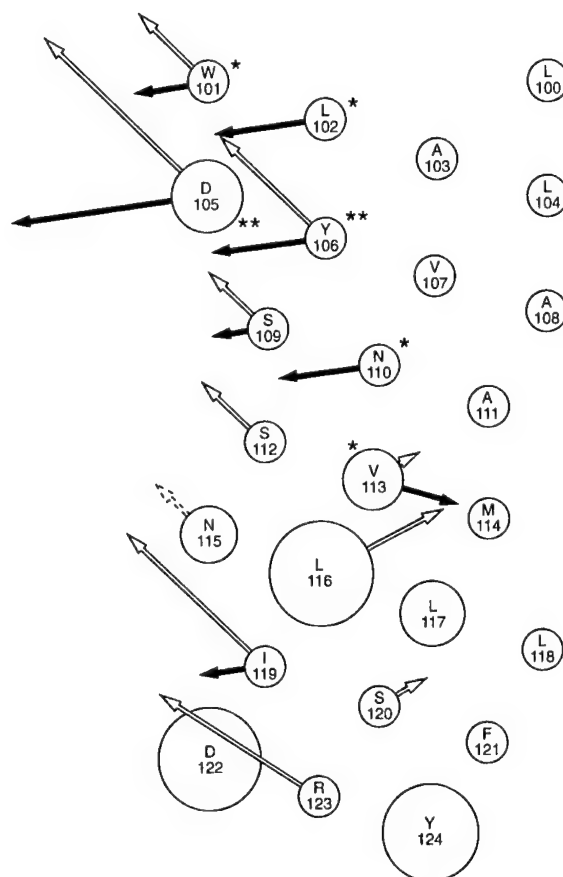


Figure 2. Scanning mutagenesis of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor. The figure shows the amino acids mutated, from Leu 100 to Tyr 124. Mutations are from X to A, or from A to G. The diameter of each circle represents the effect of the mutation on the expression level relative to the wild-type; $\text{Log} ([R_T, \text{wt}]/[R_T, \text{mutant}]) + 1$. Filled arrows represent the effect on ACh-receptor binary complex formation; $\text{Log} (K_A, \text{mutant}) - \text{Log} (K_A, \text{wild-type})$. Open arrows represent effects on ARG ternary complex formation; $\text{Log} (\alpha KKG, \text{mutant}) - \text{Log} (\alpha KKG, \text{wild-type})$. Left-pointing arrows represent a decrease relative to the wild-type, and right-pointing arrows an increase relative to the wild-type. Asterisks show a reduction in NMS affinity: *10–100-fold; **>100-fold. The dotted arrow shows a 25% reduction in catalysis of GDP-GTP exchange by the N115A mutant.

ground-state receptor structure as well as being vital for formation of the binding site.

These observations strongly support the view that this patch of amino acids is the primary agonist (and antagonist) contact site in TM3. However, they do not allow us to decide whether additional contributions to the stability of the ternary complex arise from the strengthening of their ground-state interactions with the agonist (effect on α), or from the formation of new intramolecular contacts within the receptor structure itself (effect on K) promoted by agonist and G protein binding.

Interestingly, the tightly-delimited segment of TMD 3 whose mutation affects ternary complex stability extends beyond the binding site residues to the cytoplasmic end of the helix. Mutation of S112, and most particularly, I119 and R123 all had large effects on the calculated stability of the ternary complex. Furthermore, mutation of I119 increased the Hill slope of the binding curve, and decreased the agonist K_A . Mutation of R123 also slightly steepens the ACh binding curve [8], but does not affect ACh affinity. These mutations did not affect receptor expression levels.

The results do not allow us to distinguish completely between possible intramolecular (effects on K) and intermolecular (effects on αKG) of these three mutations. Effects on α seem relatively unlikely because of their distance from the agonist binding site. On balance, it seems likely that I119 makes an intramolecular contact which is primarily important for the stability of the AR* state and the formation of the G protein binding pocket, although it probably exists in a primordial form in the AR complex. R123 may have a dual role, with potential for direct contact with the G protein, as well as intramolecular interactions.

N115 is an interesting variation on this theme, in which mutation may have slightly reduced the catalytic efficiency, rather than the stability of the AR*G complex.

Finally, there is a group containing V113, L116 and S120 whose mutation increased basal signalling, and ternary complex formation. This patch is displaced by one residue (100 degrees) from the segment which diminished signalling. Two of the mutations, V113A and L116A, also reduced receptor expression. We have argued that the primary contribution of L116 is an intramolecular interaction which promotes the stability of the ground state, but not the activated state of the receptor. A supporting part may be played by S120, whilst V113 may also have an indirect effect on the conformation of the primary binding site itself, since its mutation decreased NMS affinity while increasing ACh affinity. We postulate that these residues may help to constrain

the receptor to the inactive state in the absence of agonists.

In summary, scanning mutagenesis has revealed strong functional differentiation of the surface of TMD 3 of the M1 mAChR. It has confirmed the α -helical nature of the domain, and determined its orientation with respect to the lipid bilayer. It has shown that one corner of the helix is devoted to the formation of intermolecular (ACh-receptor, receptor-G protein) or intramolecular bonds which are essential for formation of the active AR*G ternary complex, whilst within the inner, more buried part of the domain, an adjacent compact patch of amino acids probably forms intramolecular contacts which selectively stabilise the ground state but not the activated state of the receptor. The key residues are very highly conserved in the GPCR superfamily, so these results may have wide implications for understanding the activation mechanism of these receptors.

Acknowledgments

This work was supported by the Medical Research Council (U.K.) and by the Wellcome Trust.

References

- [1] Baldwin J.M., The probable arrangement of the helices in G protein-coupled receptors, *EMBO J.* 12 (1993) 1693-1703.
- [2] Baldwin J.M., Schertler G.F.X., Unger V.M., An alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein-coupled receptors, *J. Mol. Biol.* 272 (1997) 144-164.
- [3] Berrie C.P., Birdsall N.J.M., Hulme E.C., Keen M., Stockton J.M., Solubilization and characterization of guanine nucleotide-sensitive muscarinic agonist binding sites from rat myocardium, *Br. J. Pharmacol.* 82 (1984) 853-861.
- [4] Black J.W., Leff P., Operational models of pharmacological agonism, *Proc. Roy. Soc. Lond. B.* 220 (1983) 141-162.
- [5] Clackson T., Wells J.A., A hot spot of binding energy in a hormone-receptor interface, *Science* 267 (1995) 383-386.
- [6] Farrens D.L., Altenbach C., Yang K., Khorana H.G., Requirement of rigid-body motion of transmembrane helices for light-activation of rhodopsin, *Science* 274 (1996) 768-770.
- [7] Javitch J.A., Fu D., Chen J., Karlin A., Mapping the binding site crevice of the dopamine D2 receptor by the substituted cysteine accessibility method, *Neuron* 14 (1995) 825-831.
- [8] Jones P.G., Curtis C.A.M., Hulme E.C., The function of a highly-conserved arginine residue in activation of the muscarinic M1 receptor, *Eur. J. Pharmacol.* 288, 251-257.
- [9] Kurtenbach E., Curtis C.A.M., Pedder E.K., Aitken A., Harris A.C.M., Hulme E.C., Muscarinic acetylcholine receptors: Peptide sequencing identifies residues involved in antagonist binding and disulfide bond formation, *J. Biol. Chem.* 265 (1990) 13702-13708.

- [10] Lu Z.L., Curtis C.A., Jones P.G., Pavia J., Hulme E.C., The role of the aspartate-arginine-tyrosine triad in the M1 muscarinic receptor: mutations of aspartate 122 and tyrosine 124 decrease receptor expression but do not abolish signalling, *Mol. Pharmacol.* 51 (1997) 234–241.
- [11] Page K.M., Curtis C.A.M., Jones P.G., Hulme E.C., The functional role of the binding site aspartate in muscarinic acetylcholine receptors probed by site-directed mutagenesis, *Eur. J. Pharmacol.* 289 (1995) 429–437.
- [12] Samama P., Cotecchia S., Costa T., Lefkowitz R.J., A mutation-induced activated state of the β 2-adrenergic receptor: Extending the ternary complex model, *J. Biol. Chem.* 268(1993)4625–4636.
- [13] Spalding T.A., Birdsall N.J.M., Curtis C.A.M., Hulme E.C., Acetylcholine mustard labels the binding site aspartate in muscarinic acetylcholine receptors, *J. Biol. Chem.* 269 (1994) 4092–4097.
- [14] Ward W.H.J., Timms D., Fersht A.R., Protein engineering and the study of structure-function relationships in receptors, *TIPS* 11 (1990) 280–284.
- [15] Wess J., Molecular biology of muscarinic acetylcholine receptors, *Crit. Rev. Neurobiol.* 10 (1996) 69–99.

Molecular mechanisms for the regulation of the expression and function of muscarinic acetylcholine receptors

Susan E. Hamilton, Michael L. Schlador, Lise A. McKinnon,
Renee S. Chmelar, Neil M. Nathanson*

Department of Pharmacology, Box 357750, University of Washington, Seattle, Washington 98195-7750, USA

Abstract — The regulation of muscarinic acetylcholine receptor expression and function was investigated in cultured cells and in knockout mice. Muscarinic agonist exposure causes m2 receptor desensitization and sequestration and decreases the expression of cardiac potassium channels. The expression of m2 receptors in chick retina is regulated by a developmentally regulated secreted factor. Mice lacking the m1 receptor exhibit a loss of muscarinic regulation of M-current potassium channel activity and pilocarpine-induced seizures. (©Elsevier, Paris)

Résumé — Mécanismes moléculaires de la régulation, de l'expression et de la fonction des récepteurs muscariniques à l'acétylcholine. La régulation de l'expression et de la fonction des récepteurs muscariniques a été étudiée dans des cultures de cellules et par inactivation de gènes chez la souris. Les agonistes muscariniques entraînent une désensibilisation et une séquestration des récepteurs m2 et réduisent l'expression des canaux potassium cardiaques. L'expression des récepteurs m2 dans la rétine du poulet est régulée par un facteur sécrété, lui-même contrôlé au cours du développement. Les souris 'knock out' montrent une perte de la régulation muscarinique de l'activité des canaux potassium (courant M) et présentent des arrêts cardiaques induits par la pilocarpine. (©Elsevier, Paris)

muscarinic receptor / knockout mouse / seizures / desensitization / retina / potassium channel

1. Introduction

Muscarinic acetylcholine receptors (mAChR) are expressed in neurons of the central and peripheral nervous systems, cardiac and smooth muscle, and a variety of exocrine glands. Muscarinic receptors are members of the superfamily of receptors which produce their physiological responses by interacting with members of the GTP-binding regulatory protein (G-protein) superfamily. Five subtypes of mammalian mAChR, termed m1–m5, have been identified and shown to be encoded by distinct genes. The five subtypes of mAChR can be grouped into two broad functional categories, with the m2 and m4 receptors preferentially coupling to the Gi/o family of G-proteins, and the m1, m3, and m5 receptors preferentially coupling to the Gq family of G-proteins [15]. Our laboratory has been interested in determining the molecular and cellular mechanisms involved in the regulation of the expression and function of the mAChR. In this article, we will describe work directed at clarifying the molecular mechanisms involved in the regulation of muscarinic receptor expression at the transcriptional and post-translational

levels and genetic approaches to determine the function of mAChR in vivo.

2. Regulation of mAChR by G-protein coupled receptor kinases and β -arrestin

There are both long-term and short-term mechanisms for the regulation of mAChR expression and function in response to the continued presence of acetylcholine or other agonists [11]. Phosphorylation by members of the G-protein coupled receptor kinase (GRK) family of an agonist-occupied receptor is thought to induce the binding of one of the members of the arrestin family of proteins to the activated receptor, blocking further receptor-G-protein interactions and thus causing a rapid desensitization of receptor signal transduction. In addition, the binding of arrestins to phosphorylated receptors can promote receptor internalization [3, 6].

We have described a reporter gene assay using a luciferase reporter gene under the transcriptional control of a cAMP response element (CRE) as a sensitive detector for changes in the levels of intracellular cAMP [9, 10]. This assay allows measurement of changes in physiologically relevant intracellular cAMP levels without requiring the addition of phosphodiesterase inhibitors (which are usually added

* Correspondence and reprints

when one is measuring cAMP levels biochemically) or supraphysiological concentrations of forskolin. In addition, it allows measurement of responses following cotransfection of clones encoding receptors and G-proteins in transiently transfected cells without confounding effects due to the presence of untransfected cells in the cultures. The CRE-reporter genes used in these assays when expressed in JEG-3 cells respond only to changes in cAMP and not to intracellular calcium. Furthermore, the changes in CRE-luciferase expression require the presence of a functional cAMP-dependent protein kinase. This system allows transient transfection experiments to be carried out using a virtually unlimited combination of receptors, G-proteins, kinases, etc. Our studies have shown that this approach can be used to study the function of both muscarinic receptors and G-proteins. [9, 10]. We have now used this system to determine the ability of G protein-coupled receptor kinase-2 (GRK2) and β -arrestin-1 to regulate the phosphorylation, desensitization, and sequestration of the m2 mAChR [12]. Treatment of JEG-3 cells transiently expressing an epitope-tagged m2 mAChR with the muscarinic agonist carbachol induced an approximately 4-fold increase in receptor phosphorylation in the absence and an 8-fold increase in receptor phosphorylation in the presence of cotransfected GRK2, when compared to cells transfected with receptor alone in the absence of agonist. Using the expression of a cAMP-regulated reporter gene to measure receptor function, in the absence of cotransfected GRK2 and β -arrestin, the m2 mAChR exhibited modest functional desensitization of its ability to regulate CRE-luciferase expression following exposure to agonist. While transfection of β -arrestin-1 by itself had no effect on m2 signaling, transfection of GRK2 enhanced agonist-induced desensitization, and cotransfection of GRK2 and β -arrestin-1 acted synergistically to promote functional desensitization. GRK-2 and β -arrestin-1 did not affect signaling by a receptor deletion mutant lacking the GRK phosphorylation sites in the third cytoplasmic loop. GRK2 and β -arrestin-1 also synergistically increased both the rate and magnitude of agonist-induced sequestration of the m2 mAChR. These results demonstrate that GRK2 and β -arrestin-1 act in concert to cause agonist-induced desensitization and sequestration of the m2 mAChR and show that these proteins have multiple actions on the m2 mAChR.

3. Regulation of GIRK potassium channels by mAChR activation

Muscarinic receptors decrease the rate of contraction of the heart in part by activating a G-protein

coupled inwardly rectifying potassium channel, composed of two subunits, GIRK1 and GIRK4 [4]. Muscarinic receptors activate GIRK channels in atrial cells but not in ventricular cells. In chick heart, this tissue-specific functional response is due to the fact that, while atrial cells express both GIRK1 and GIRK4, ventricular cells only express significant levels of GIRK1 but not GIRK4, resulting in an inability to form a heteromultimeric functional channel [14]. We have previously demonstrated that long-term (i.e., over several hours) exposure of chick heart cells to muscarinic agonists causes large decreases in the number of muscarinic receptor binding sites and in the levels of mRNA encoding the mAChR [1]. We have recently investigated the effects of mAChR activation on the expression of GIRK subunits [14]. Treatment of chick embryos in ovo with the muscarinic agonist carbachol resulted in a time- and concentration-dependent decrease in the level of GIRK1 and GIRK4 mRNAs in the atria. Carbachol treatment also decreased the level of GIRK1 polypeptide expression as detected by immunoblot analysis. The decrease in mRNAs could be prevented by concomitant administration of the antagonist atropine, and subsequent administration of atropine after treatment with carbachol alone resulted in a gradual return of GIRK1 and GIRK4 mRNAs back to control levels. These results demonstrate that persistent activation of the mAChR can decrease the expression of an effector protein required for receptor-mediated signaling. Because a variety of G-protein coupled receptors can regulate GIRK activity in the nervous system, these results raise the possibility that this could represent a novel mechanism for the regulation of neurotransmitter receptor responsiveness by both homologous and heterologous receptor activation.

While agonist treatment decreased the levels of GIRK1 and GIRK4 mRNAs in atria, there was no effect on the level of GIRK1 mRNA in the ventricles. In contrast, carbachol treatment causes similar decreases in the levels of mAChR mRNAs and mAChR binding sites in atria and ventricles. These results suggest that the signaling mechanisms for the regulation of mAChR mRNAs levels following mAChR activation are different from those responsible for the regulation of GIRK mRNA levels.

4. Regulation of retinal mAChR during embryonic development

Previous working using affinity alkylation and SDS gel electrophoresis suggested that the pattern of expression of mAChR in the chick retina changes dramatically during embryonic development [5, 13].

We used solution hybridization, immunoprecipitation, and immunoblot analyses to examine the expression of chick m2 (cm2), cm3, and cm4 mAChR mRNA and protein in embryonic and post hatched chick retina. While the cm4 receptor is the predominant receptor early in development, the cm3 receptor increases somewhat and the cm2 receptor increases particularly dramatically during the second week of embryonic development [8]. A similar increase in cm2 expression occurs in cultured embryonic chick retinal cells. Treatment of fresh cultured retinal cells with conditioned medium from retinal cells cultured for at least 5 days, but not from retinal cells cultured for 1–2 days, results in a selective increase in expression of cm2 but not cm3 or cm4 receptors due to the action of a protease-sensitive secreted factor [7]. The cm2-inducing factor stimulates expression of a luciferase reporter gene under the transcriptional control of the cm2 promoter, showing that the increase in cm2 expression results from increased gene transcription. Addition of 14 known neurotrophic and growth factors to cultured retinal cells did not increase the expression of the cm2 receptor. The induction of the cm2 receptor gene in the retina may be due to the action of a novel developmentally regulated secreted factor.

5. Genetic dissection of mammalian mAChR function

We have begun to use gene targeting by homologous recombination to determine the function of individual mAChR subtypes in the mammalian nervous system [2]. A targeting vector was designed to delete the translational start site and the region encoding the amino terminal, first transmembrane domain, and a portion of the first cytoplasmic loop from the mouse m1 receptor gene. Embryonic stem cell clones in which the mutant gene was incorporated into the genome by homologous recombination were used to obtain m1 knockout mice. Homozygous m1 mutant mice arose from crosses of heterozygotes with a Mendelian ratio of approximately 1:4, and exhibited no differences in body weight, longevity, fertility, or overt behavior compared to wild type mice.

There was an approximately 50% decrease in the total number of mAChR binding sites from the forebrains of knockout compared to wild type mice, while there was no significant decrease in the level of mAChR sites in the cerebellum (where there is little m1 receptor expressed). Immunoprecipitation analyses demonstrated that there was no detectable expression of m1 receptor in knockout mice, while the levels of m2, m3, and m4 receptors were not

affected. Immunocytochemical analyses showed that there was also no significant difference in the overall morphology of the brain or in the pattern of expression of the m2, m3, and m4 receptors.

Muscarinic agonists cause robust suppression of the M-current potassium channel in sympathetic neurons derived from wild type mice, while there was no effect of muscarinic agonists on the M-current in sympathetic neurons from knockout mice. In contrast, angiotensin II was able to suppress the M-current in both wild type and mutant neurons. Thus, the m1 receptor is the only muscarinic receptor which regulates M-current activity in sympathetic neurons.

Systemic administration of the muscarinic agonist pilocarpine causes seizures that resemble those observed in patients with temporal lobe epilepsy. Homozygous mutant mice are totally resistant to the administration of doses of pilocarpine which result in multiple tonic-clonic seizures in wild type mice. Interestingly, heterozygous mutant mice are almost as resistant as knockout mice to the seizures produced by administration of high levels of pilocarpine. These results suggest that the m1 receptor plays a crucial role in the initiation of seizures in the pilocarpine model of epilepsy. The m1 mutant mice may be useful in delineating the pathways involved in seizure initiation and in the roles of the m1 receptor in mediating the many behavioral responses elicited by acetylcholine in the nervous system.

References

- [1] Habecker B.A., Nathanson N.M., Regulation of muscarinic acetylcholine receptor mRNA expression by activation of homologous and heterologous receptors, *Proc. Natl. Acad. Sci. USA* 89 (1992) 5035–5038.
- [2] Hamilton S.E., Loose M.D., Qi M., Levey A.I., McKnight G.S., Hille B., Idzerda R.L., Nathanson N.M., Disruption of the m1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice, *Proc. Natl. Acad. Sci. USA* 94 (1997) 13311–13316.
- [3] Goodman O. B. Jr., Krupnick J.G., Santini F., Gurevich V.V., Penn R.B., Gagnon A.W., Keen J.H., Benovic J.L., Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor, *Nature* 383 (1996) 447–450.
- [4] Krapivinsky G., Gordon E.A., Wickman K., Velimirovic B., Krapivinsky L., Clapham D.E., The G-protein-gated atrial K⁺ channel I_{KACH} is a heteromultimer of two inwardly rectifying K⁺-channel proteins, *Nature* 374 (1995) 135–141.
- [5] Large T.H., Rauh J.J., DeMello F.G., Klein W.L., Two molecular weight forms of muscarinic acetylcholine receptors in the avian central nervous system: switch in predominant form during differentiation of synapses, *Proc. Natl. Acad. Sci. USA* 82 (1985) 8785–8789.
- [6] Lohse M.J., Andexinger S., Pitcher J., Trukawinski S., Codina J., Faure J.-P., Caron M.G., Lefkowitz R.J., Receptor-

- specific desensitization with purified proteins, *J. Biol. Chem.* 267 (1992) 8558–8564.
- [7] McKinnon L.A., Gunther E.C., Nathanson N.M., Developmental regulation of the cm2 muscarinic acetylcholine receptor gene: Selective induction by a secreted factor produced by embryonic chick retinal cells, *J. Neurosci.* 18 (1998) 59–69.
- [8] McKinnon L.A., and Nathanson N.M., Tissue-specific regulation of muscarinic acetylcholine receptor expression during embryonic development, *J. Biol. Chem.* 270 (1995) 20636–20642.
- [9] Migeon J.C., Nathanson N.M., Differential regulation of cAMP-mediated gene expression by m1 and m4 muscarinic acetylcholine receptors: Preferential coupling of m4 receptors to $G_{i\alpha-2}$, *J. Biol. Chem.* 269 (1994) 9767–9773.
- [10] Migeon, J.C., Thomas, S., and Nathanson, N.M., Regulation of cAMP-mediated gene expression by wildtype and mutant G-proteins: Inhibition of adenylyl cyclase by muscarinic receptor-activated and constitutively activated $G_{o\alpha}$, *J. Biol. Chem.* 269 (1994) 29146–29152.
- [11] Nathanson N.M., Regulation and development of muscarinic acetylcholine number and function, in: Brown J.H. (Ed.), *The Muscarinic Receptors*, The Humana Press, Clifton, N.J., 1989, pp. 419–454.
- [12] Schlador M.L., Nathanson N.M., Synergistic regulation of m2 muscarinic acetylcholine receptor desensitization and sequestration by G-protein-coupled receptor kinase-2 and β -arrestin-1, *J. Biol. Chem.* 272 (1997) 18882–18890.
- [13] Skorupa A.F., Klein W.L., Developmentally regulated secreted factors control expression of muscarinic receptor subtypes in embryonic chick retina, *J. Neurochem.* 60 (1993) 2087–2097.
- [14] Thomas L.S., Chmelar R.S., Lu C., Halvorsen S.W., Nathanson N.M., Tissue specific regulation of G-protein coupled inwardly rectifying K^+ channel expression by muscarinic receptor activation in ovo, *J. Biol. Chem.* 272 (1997) 29958–29962.
- [15] Wess J., Molecular biology of muscarinic acetylcholine receptors, *Crit. Rev. Neurobiol.* 10 (1996) 69–99.

Role of neurotrophic factors in motoneuron development

Christopher E. Henderson, Yoichi Yamamoto, Jean Livet,
Vilma Arce, Alain Garcès, Odile deLapeyrière

INSERM U382, IBDM (CNRS-INSERM-Univ. Méditerranée-AP Marseille), Campus de Luminy, 13288 Marseille cedex 09, France

Abstract — More than 10 factors from different gene families are now known to enhance motoneuron survival, and to be expressed in a manner consistent with a role in regulating motoneuron numbers during development. We provide evidence that: a) different factors may act on different sub-populations of motoneurons; b) different factors may act in synergy on a given motoneuron. Thus, the functional diversity of motoneurons, and the cellular complexity of their environment, may be reflected in the mechanisms that have evolved to keep them alive. ©Elsevier, France)

Résumé — **Rôle des facteurs neurotrophiques au cours du développement des motoneurones.** Plus de 10 facteurs de familles géniques différentes sont maintenant connus pour leur capacité à favoriser la survie des motoneurones. Le profil d'expression de ces facteurs est cohérent avec l'idée qu'ils sont impliqués dans la régulation du nombre de motoneurones au cours du développement. Nos résultats suggèrent que : a) différents facteurs peuvent agir sur des sous-populations de motoneurones différentes; b) différents facteurs peuvent agir en synergie sur un motoneurone donné. Donc, la diversité fonctionnelle des motoneurones, ainsi que la complexité cellulaire de leur environnement, se retrouvent dans les mécanismes qui ont évolué pour assurer leur survie. ©Elsevier, France)

motoneuron / neurotrophic / survival / neurogenerative disease

1. Introduction

Synapses formed on muscle by motoneurons are of vital importance for the survival of the organism. This means that the survival of these neurons and the maintenance of their synapses are the object of multiple controls [2]. Another consequence is that diseases that affect the survival of motoneurons are rapidly fatal. Molecules that keep motoneurons alive during development may therefore be useful in man to slow down pathological neuronal degeneration [4].

Neurotrophic factors are a diverse group of polypeptides with multiple biological roles; their common feature is that they promote survival of certain neuronal populations [5]. Certain of these factors act during development to regulate neuron numbers in the peripheral nervous system. Since the classical limb ablation experiments of Viktor Hamburger in the chick embryo, it has been known that motoneurons too require neurotrophic factors from the limb for their normal survival, but only recently have some of the molecules potentially involved been identified [2, 5]. However, although many candidate factors are known to keep motoneurons alive in experimental situations, the physiological survival factors for motoneurons have not yet been unambiguously identified. Furthermore, the number and diversity of the candidate factors identified are still not well understood in biological terms.

2. Identification of motoneuron survival factors

Our approach to the identification of molecules potentially involved in keeping motoneurons alive *in vivo* has been to develop highly purified preparations of spinal motoneurons from chicken and rat embryos [6]. When cultured in complete media, these neurons will nevertheless undergo apoptosis unless provided with exogenous trophic support, either in the form of tissue extracts or conditioned media or, more importantly, in the form of recombinant neurotrophic factors. Although this culture system is obviously in some ways distant from the *in vivo* situation, it has clear advantages: molecules thus identified must be acting directly on motoneurons, and their potency and efficacy can be directly and precisely quantified. Indeed, many of the factors identified in this system act extremely potent, and can provide long-term support for a significant fraction of the motoneurons initially seeded.

The factors identified belong to several different gene families: i) neurotrophin family: BDNF (brain-derived neurotrophic factor), NT-3 (neurotrophin-3), NT-4/5 [7]; ii) transforming growth factor-beta family: GDNF (glial cell line-derived neurotrophic factor), NTN (neurturin), PSP (persephin), TGFβ3 [3, 8–10]; iii) cytokines of the interleukin-6 family: CNTF (ciliary neurotrophic factor), LIF (leukaemia inhibitory factor), CT-1 (cardiotrophin-1) [1, 8, 11]; and iv) other growth factors: members of the FGF

(fibroblast growth factor) family, HGF (hepatocyte growth factor) [3, 13].

Nearly all of these factors have also been shown by others to be active in preventing motoneuron cell death *in vivo*, either during programmed cell death in the chicken or mouse embryo, or following axotomy in neonatal rat pups, or in mouse mutant strains that show motoneuron degeneration. Thus, cultured purified motoneurons have provided a reliable testing system, and there is a general consensus that many factors can indeed act to promote motoneuron survival. One consequence of this is that they are all potential candidates for therapeutic applications involving motoneuron degeneration. However, much remains to be learned about their biological roles. Are all indeed required for normal motoneuron survival? If so, why are so many different factors involved? And at what stages? We and others have been addressing different aspects of this problem.

3. Trophic requirements of motoneuron sub-populations

In terms of their early development, embryonic motoneurons have often been considered as a homogeneous group of cells. However, different classes of motoneuron (alpha, gamma, fast, slow, etc.) fulfill very different functions in the adult. Furthermore, it is known that different sub-populations of embryonic motoneurons express different molecular markers (e.g., transcription factors). It is therefore likely that the development of these sub-populations may be differentially regulated.

For instance, heterogeneity is apparent in terms of the action of neurotrophic factors on the survival of developing motoneurons. In knockout mice for the neurotrophic factor GDNF or for the cytokine receptors CNTFR α or LIFR β , motoneuron loss is reproducible but partial. This is mirrored by the observation that no individual neurotrophic factor has been shown to be capable of keeping all motoneurons alive for long periods, and that combinations of factors are more efficient [1, 11]. These results may be compared with well-established data on the development of spinal sensory ganglia, in which sub-populations of neurons with different functions (proprioceptive, nociceptive, etc.) respond to, and require, specific neurotrophic factors for their survival. What is lacking in the case of motoneurons is a suitable repertoire of molecular markers for motoneurons responding to different factors, and a direct link between molecular diversity in the embryo and functional diversity in the adult.

One obvious potential marker for responsiveness to a given neurotrophic factor is the presence of the

appropriate receptor. Although certain receptors for neurotrophic factors are expressed by virtually all motoneurons, this is not the case for all of them. Probably the best example is provided by c-Met, the receptor for hepatocyte growth factor (HGF/SF). HGF/SF is an important component of myotube-conditioned media, as antibodies to HGF block 65% of the trophic activity of such media for cultured motoneurons [13]. However, although its actions on cultured motoneurons are quite potent, HGF is unable to keep all of the motoneurons alive, even at short survival times. This is mirrored by the fact that at a given rostral-caudal level, only a fraction of the motoneurons express c-Met. Thus, although its role *in vivo* remains to be demonstrated, HGF may play a role in maintaining a specific sub-population of motoneurons.

4. Synergistic actions of neurotrophic factors

Another possibility is that each cellular partner of a given motoneuron may interact with the motoneuron using a different factor. For example, we have shown that GDNF is produced by Schwann cells, whereas the cytokine CT-1 is synthesized by muscle [8, 11]. Blockade of GDNF activity reduces the levels of trophic support produced by Schwann cells by 75%, while antibodies to CT-1 inhibit muscle cell line-conditioned media by 45%. We recently showed that these factors can act in synergy on motoneuron survival, providing a potential mechanism for selective preservation during development of those motoneurons that have contacted both the target muscle and Schwann cells [1]. This research may have direct repercussions for the use of neurotrophic factors in the clinic, and indeed there are several reports in animal models of increased effectiveness of the administration of combinations of neurotrophic factors.

5. Conclusions

The field of study of survival factors for motoneurons has seen rapid progress over the past 4 years. Many of the potential actors (neurotrophic factors, receptors, etc.) have now been identified, and new model systems have been developed to test their activities. Nevertheless, we are only just beginning to fit them into the puzzle represented by the development over time of the different components of the neuromuscular system [2, 12]. In particular, we believe that it is important to appreciate the cellular complexity of the motoneuron population in order to understand the action of neurotrophic factors in

this system. This may be of importance not only for developmental studies, but also in evaluating candidate trophic factors for potential clinical use in specific pathological situations.

Acknowledgments

Studies from our laboratory were supported by INSERM, CNRS, Association Française contre les Myopathies (AFM), Institut pour la Recherche sur la Moelle Epinière (IRME), and European Commission BIO4 contract CT960433.

References

- For brevity, only recent work and reviews from our laboratory are given. Many important articles by others in the field are cited in the listed references.
- [1] Arce V., Pollock R.A., Philippe J.M., Pennica D., Henderson C.E., deLapeyrière O., Synergistic effects of Schwann- and muscle-derived factors on motoneuron survival involve GDNF and CT-1, *J. Neurosci.* 18 (1998) 1440–1448.
 - [2] deLapeyrière O., Henderson C.E., Motoneuron differentiation, survival and synaptogenesis, *Curr. Opin. Genet. Dev.* 7 (1997) 642–650.
 - [3] Gouin A., Bloch-Gallego E., Tanaka H., Rosenthal A., Henderson C.E., Transforming growth factor-beta 3, glial cell line-derived neurotrophic factor, and fibroblast growth factor-2, act in different manners to promote motoneuron survival in vitro, *J. Neurosci. Res.* 43 (1996) 454–464.
 - [4] Henderson C.E., Neurotrophic factors as therapeutic agents in amyotrophic lateral sclerosis. Potential and pitfalls, *Adv. Neurol.* 68 (1995) 235–240.
 - [5] Henderson C.E., Role of neurotrophic factors in neuronal development, *Curr. Opin. Neurobiol.* 6 (1996) 64–70.
 - [6] Henderson C.E., Bloch-Gallego E., Camu W., Purified embryonic motoneurons, in: Cohen J., Wilkin G., (Eds.), *Nerve cell culture: a practical approach*, Oxford University Press, London, 1995, pp. 69–81.
 - [7] Henderson C.E., Camu W., Mettling C., Gouin A., Poulsen K., Karihaloo M., Rullamas J., Evans T., McMahon S.B., Armanini M.P., Berkemeier L., Phillips H.S., Rosenthal A., Neurotrophins promote motor neuron survival and are present in embryonic limb bud, *Nature* 363 (1993) 266–270.
 - [8] Henderson C.E., Phillips H.S., Pollock R.A., Davies A.M., Lemeulle C., Armanini M., Simpson L.C., Moffat B., Vandlen R.A., Koliatsos V.E., Rosenthal A., GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle, *Science* 266 (1994) 1062–1064.
 - [9] Klein R.D., Sherman D., Ho W.H., Stone D., Bennett G.L., Moffat B., Vandlen R., Simmons L., Gu Q., Hongo J.A., Devaux B., Poulsen K., Armanini M., Nozaki C., Asai N., Goddard A., Phillips H., Henderson C.E., Takahashi M., Rosenthal A., A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor, *Nature* 387 (1997) 717–721.
 - [10] Milbrandt J., de Sauvage F., Fahrner T.L., Baloh R.H., Leitner M.L., Tansey M.L., Lampe P.A., Heuckeroth R.O., Kotzbauer P.T., Simburger K.S., Golden J.P., Davies J.A., Vejsada R., Kato A.C., Hynes M., Sherman D., Nishimura M., Wang L.-C., Vandlen R., Moffat B., Klein R.D., Poulsen K., Gray C., Garces A., Henderson C.E., Phillips H.S., Johnson E.M., Persephin, a novel neurotrophic factor related to GDNF and Neurturin, *Neuron* 20 (1998) 245–253.
 - [11] Pennica D., Arce V., Swanson T.A., Vejsada R., Pollock R.A., Armanini M., Dudley K., Phillips H.S., Rosenthal A., Kato A.C., Henderson C.E., Cardiotrophin-1, a cytokine present in embryonic muscle, supports long-term survival of spinal motoneurons, *Neuron* 17 (1996) 63–74.
 - [12] Pettmann B., Henderson C.E., Neuronal cell death, *Neuron* 20 (1998) 633–647.
 - [13] Yamamoto Y., Livet J., Pollock R.A., Garces A., Arce V., deLapeyrière O., Henderson C.E., Hepatocyte growth factor (HGF/SF) is a muscle-derived survival factor for a subpopulation of embryonic motoneurons, *Development* 124 (1997) 2903–2913.

Cholinergic foundations of Alzheimer's disease therapy

Ezio Giacobini

HUG, Belle-Idée, Department of Geriatrics, University Hospitals of Geneva, route de Mon-Idée, 1226 Thonex, Geneva, Switzerland

Abstract — Cholinesterase inhibitors (ChEI) represent the drug of choice for Alzheimer's disease (AD) treatment. They produce significant improvement on cognitive as well as non-cognitive function for a period up to 1 year during the first 3 years following clinical diagnosis. The magnitude of cognitive improvements is similar for different ChEI, however, differences are seen with regard to incidence and severity of side effects, optimal ChE inhibition, pharmacokinetic properties and mode of administration. (©Elsevier, Paris)

Résumé — **Fondement cholinergique du traitement de la maladie d'Alzheimer.** Les inhibiteurs de la cholinestérase constituent le traitement de choix de la maladie d'Alzheimer. Prescrits dans les trois premières années suivant le diagnostic clinique, ils améliorent de façon significative jusqu'à un an les fonctions cognitives et non cognitives. L'ampleur des améliorations cognitives est semblable pour les divers inhibiteurs de la cholinestérase, mais des différences sont observées au niveau de l'incidence et de la gravité des effets secondaires, de l'inhibition optimale de la cholinestérase, de la pharmacocinétique et du mode d'administration. (©Elsevier, Paris)

cholinesterase inhibitors / Alzheimer's disease

1. Past and future of cholinesterase inhibitors in Alzheimer's therapy

Cholinesterase inhibitors (ChEI) represent the drugs of choice for Alzheimer's disease (AD) treatment. Following the first generation of non-specific drugs such as physostigmine, a second generation of more selective products has been developed. These compounds show less side effects in humans at effective doses. The data originating from recent clinical trials demonstrate that optimization of effect and maintenance of clinical gains for 1 year is presently a feasible target. Future studies will demonstrate whether or not: i) if it is possible to extend the benefit for a period longer than 12 months; and ii) if there is a protective effect for minimal cognitive impairment (MCI) subjects at risk for conversion to AD. The considerable amount of basic knowledge on the cholinergic system's physiology and pharmacology accumulated during the last 50 years has greatly contributed the therapeutic success of ChEI. Physostigmine was first isolated and used in therapy more than half a century before the discovery of acetylcholine as a neurotransmitter [9] (*table I*). Cholinesterases were isolated from the mammalian brain 20 years after the discovery of ACh as a neurotransmitter (*table I*). The utilization of ChEI as anti-Alzheimer drugs represents the most recent acquisition of the CNS drugs development which started in the fifties with neuroleptics and continued into the sixties with the discovery of anti-Parkinson agents. The first ChEI to be used against AD was physostigmine followed by tacrine (*table I*). Subsequently, metrifonate and galanthamine were tested simultaneously

with other ChEIs (*table I*). Cholinesterase inhibitors, particularly s.c. second generation, (post-physostigmine and post-tacrine compounds), affect cortical as well as sub-cortical neurotransmitters other than ACh [14]. Their effects on NE and DA are of particular clinical interest for AD therapy. Another feature of ChEI is their ability to enhance the release of non-amyloidogenic soluble derivatives of APP from cortex both in vitro and in vivo and possibly

Table I. Cholinesterase inhibitors. a long history.

	<i>Ref</i>
Physostigmine	Isolation 1864 (Jobst and Hesse) ^a Therapeutical use 1877 (Laquer) ^a
Acetylcholine	Synthesis 1867 (Baeyer) A neurotransmitter 1914 [9] ^a
Cholinesterases	Isolation and name 1932 (Stedman) ^a Found in the brain 1935 (Stedman and Stedman) ^a
Cholinesterase inhibitors as anti-Alzheimer drugs	1979 Physostigmine i.v. [10] 1983 Physostigmine [33] 1981 Tacrine [32] 1988 Metrifonate [3] 1989 Galanthamine [28]

^aGoodman and Gilman, *The Pharmacological Basis of Therapeutics*, McGraw Hill Publ., Ninth Edition.

to slow down formation of amyloidogenic compounds in AD brain [24]. This process could explain the stabilization effect on cognitive deterioration seen during ChEI therapy. Cholinergic alternatives other than use of ChEI are being investigated such as the utilization of nicotinic and muscarinic agonists. Non-selectivity and presence of severe side effects has so far prevented therapeutic application of these compounds as complementary cholinergic approaches.

2. Second generation cholinesterase inhibitors

Cholinesterase inhibitors presently in clinical trials or in current use (1998) in Japan, USA and Europe include less than 10 drugs. Most of these compounds have advanced to clinical phase III and IV and two (tacrine, and donepezil) are registered in USA and in Europe [16]. Two other compounds (rivastigmine and metrifonate) are close to registration both in USA and Europe. This second generation ChEI, in order to replace tacrine, has to fulfill specific requirements such as lower toxicity and easier administration [16]. Based on current selection criteria and occurrence of complications a number of ChEIs (particularly carbamates) has been discontinued. Are there major differences among various compounds with regard to efficacy, percentage of treatable patients, drop-out and incidence of side effects? *Table II* compares the effect of six ChEIs on ADAS-cog test using intention to treat (ITT) criteria. The duration of these clinical trials varied from 24 to 30 weeks and the total number of patients was above 4000. All six ChEIs (tacrine, eptastigmine, donepezil, rivastigmine, metrifonate and galanthamine) produced statistically significant improvements using standardized and internationally validated measures of both cognitive and non-cognitive function. The magnitude of cognitive effects

measured with the ADAS-cog scale for all six drugs either expressed as a difference between drug- and placebo-treated patients or as the difference between drug-treated patients and baseline is similar (*table II*). This similarity in size of cognitive improvement suggests a ceiling effect at approximately five ADAS-cog points average for ChEI tested in mild to moderate (CDR 1–1.5) stages of the disease at present dosage. These results suggest that a maximal effect has not yet been reached with all drugs tested. The high percentage of drop-outs and more severe side-effects seen with some particular drugs such as tacrine (hepatotoxicity and general cholinergic toxicity) suggest a limit in achievable levels of ChE inhibition and demonstrable drug effect. Similarity in clinical efficacy is also underlined by the practically identical effect on global scales such as the clinicians interview-based impression of change-plus (CIBIC-plus) of a 0.4 points difference seen for tacrine, rivastigmine and metrifonate, drugs having very different chemical structure and pharmacological profile. The percentage of improved patients varies from 25% (rivastigmine low dose) to 50% (tacrine and donepezil high dose) with an average of 34%. This indicates that one-third of treated patients shows a positive response, however, one should keep in mind that a small percentage (about 10%) of patients do not improve on the ADAS-cog with any of the used drugs while other patients demonstrate a response significantly higher than five points. Difference between responders versus non-responders may reflect the level of cholinergic damage present in the brain and/or a genetic factor (e.g., APOE-4 alleles). From the 6-month data presented in *table II* one can also observe that patients treated with the active compound change little cognitively from baseline at the beginning of the study to the end. This suggests a stabilization effect on patient deterioration which could be clinically more significant than the expected symptomatic improvement.

Table II. The effect of six cholinesterase inhibitors on ADAS-cog test (ITT).

Drug (ref)	Dose (mg/day)	Duration of study (weeks)	Treatment difference from		Improved patients (%)	Drop-out (%)	Side effects (%)
			Placebo*	Baseline**			
Tacrine [12, 21]	120–160	30	4.0–5.3	0.8–2.8	30–50	55–73	40–58
Eptastigmine [7, 20]	45	25	4.7	1.8	30	12	35
Donepezil [11, 29, 30]	5–10	24	2.8–4.6	0.7–1	58	5–13	6–13
Rivastigmine [1]	6–12	24	1.9–4.9	0.7	25	15–36	28
Metrifon [5, 6, 23, 25]	25–75–80	12–26	2.8–3.1–3.2	0.75–0.5	35	2–21–8	2–12
Galanthamine [36]	30	12	3.3	1.8	–	–33	–

ADAS-cog, AD assessment scale-cognitive subscale; ITT, intention to treat; *study end point vs. placebo; **study end point vs. baseline, donepezil E 2020; ENA 713, rivastigmine; metrifon, metrifonate.

3. Differences in effects among cholinesterase inhibitors

The relation between percentage of peripheral ChE inhibition and cognitive (ADAS-cog) or global impression of change rated by the clinician (CGIC) effect is reported in *table III*. These data support the fundamental principle of cholinergic pharmacology that brain ChE inhibition relates directly to an increase of synaptic ACh levels leading to cognitive and memory improvement. This relationship might vary for each drug as each compound may produce cognitive improvement and therapeutic effect at different levels of ChE inhibition [13, 16, 18]. This concept is in agreement with pharmacological data in animals [16, 22] and humans [13]. The level of peripheral enzyme inhibition measured in the patient (AChE activity in erythrocytes or plasma BuChE activity) varies between 30% and 80% (*table III*) depending on dose and pharmacological characteristics of the compound. The ChE inhibition measured in the periphery (plasma or RBC) does not accurately reflect the level of CNS inhibition. For some drugs (donepezil and metrifonate) the mean level of peripheral ChE inhibition is 65–70% and could be brought to be as high as 90%. For other drugs, inhibition is as low as 30% (physostigmine, tacrine, eptastigmine). As predicted by pharmacological and behavioral data, there is a direct correlation between ChE inhibition (or drug plasma concentration) and cognitive effect [14, 15]. Also, as expected from animal models, there is no direct correlation between ChE inhibition and severity of side effects [2, 29]. As an example, degree of improvement in ADAS-cog and CIBIC-plus was directly proportional with the degree of AChE (RBC) inhibition following metrifonate treatment while side effects were very mild even at high level of inhibition (70%) [8]. A direct clinical implication of this relationship is that drugs producing only mild cholinergic side effects at high dosage (resulting in high level of brain ChE inhibition) may be tested in the patient within their full range of therapeutic potential. The predicted U-sha-

ped curve of the relation ChE inhibition/cognitive effect can be observed for some ChEI at a certain dose range (*table III*). This is explained by the fact that increasing the dose of the inhibitor, efficacy is progressively increased until adverse effects become a limiting factor. A second explanation is the specific inhibition kinetic of ChEI and the substrate-induced saturation effect of ChEs (substrate inhibition effect). Under normal conditions, the level of ACh brain elevation varies according to brain AChE inhibition [16–18]. With increased brain concentrations of ACh, substrate inhibition kinetics of enzyme activity become a limiting factor. This relation is observed in brain tissue in vitro and is probably present also in vivo [17]. Plotting velocity of enzymatic reaction against substrate (ACh) concentration, a bell-shaped curve with a defined peak in case the of AChE activity in brain and erythrocytes and a sigmoid curve in case of BuChE (butyrylcholinesterase) activity in serum are observed. Thus, AChE is inhibited by a large excess of ACh such as it can be produced by a high level of inhibition of brain AChE. Substrate elevation decreases the catalytic potency of the enzyme and subsequently its pharmacological effect. From this relationship it can be predicted that high ChE inhibition reached too rapidly in time during treatment because of a rapid passage of the drug into the brain and its accumulation in CNS will not further increase efficacy but only augment CNS dependent side effects (drowsiness, nausea, vomit, etc.). Therefore, therapeutically, it is an advantage to use a slow-release type of ChEI inhibiting both brain enzymes (AChE and BuChE) at a slow pace (using escalating dose followed by a maintenance dose) in order to reach gradually steady-state levels of brain ACh. Such a procedure may also lower the risk of cholinergic receptor down-regulation and tolerance-producing enzyme induction. There is an excellent agreement between clinical and animal data for both physostigmine and tacrine with regard to dose/behavioral effects relationships. Rupniak et al. [31] using two primate models in rhesus monkeys found that both tacrine and physostigmine improved

Table III. Relation between percent ChE inhibition and effect on ADAS-cog or CGIC.

Drug (ref)	Dose (mg/day)	Steady state (% inhibition)	Optimal ChE (% inhibition)	Correlation ChEI/ADAS-cog or CGIC
Physostigmine [34]	3–16	40–60 (BuChE)	30–40	U-shaped
Eptastigmine [7, 19]	30–60	13–54 (AChE)	30–35	U-shaped
Metrifonate [4]	30	35–75 (AChE)	65–80	U-shaped
Donepezil [29]	5	64 (AChE)	60	Linear
Tacrine [12, 21]	160	40 (BuChE) 60 (AChE)	30	Linear
Galanthamine [36]	20–50	50–60 (AChE)	50	U-shaped

ADAS-cog, A D assessment scale-cognitive subscale; CGIC, clinician global impression of change.

Table IV. Comparison of pharmacokinetic properties of five cholinesterase inhibitors used in Alzheimer therapy.

Drug ref.	Plasma conc. ($\mu\text{g/L}$) <i>C-max</i>	Time to peak (h) <i>T-max</i>	Elim. half- life (h) $T_{1/2}$	Metabolism type	Plasma prot. % abs.	Elimination (%)	Bio- availability (%)
Tacrine	—	1–2	2–4	Hepatic (P450)	55	—	17
Donepezil [30]	30–60	3–4	73	Hepatic (P450)	96	60 renal	100
Rivastigmine [1]	—	0.3	5	Non-hepatic	40	Renal	36
Metrifonate [27]	500	0.5	2	Non-hepatic	20	Renal	90
Galanthamine [35]	543	0.5	4.4–5–7	Hepatic (P450)	10	Renal	100

visual recognition memory significantly. Both drugs showed a clear inverse U-shaped relationship with a maximal effect at around 0.010–0.02 mg/kg i.m. for physostigmine and 0.8–1 mg/kg for tacrine. Lower or higher doses did not improve performance but only side effects. Central cholinergic side effects which may develop early in the treatment are not related directly to brain AChE inhibition but mainly to elevation of ACh levels [17, 18]. In the rat, this increase in ACh is not directly correlated to AChE inhibition. Peripheral side-effects may occur depending on a rapid redistribution of the drug (or of its metabolites) between non-CNS (peripheral organs such as bronchi, gastrointestinal tract, heart and muscles) and CNS compartments and peripheral (BuChE) inhibition. The occurrence of both peripheral and central side effects seen with all compounds tested so far demonstrates that none of the presently available inhibitors is truly 'brain specific'. With very long-acting ChEI (such as methanesulfonyls and organophosphates) the rate of enzyme activity recovery is limited by the rates of synthesis of new enzyme. This rate of synthesis is different for each tissue. In rat brain, the synthesis of new enzyme occurs with a mean half-life of approximately 11 days compared to 1, 3, and 6 days for ileum, heart and muscles [26]. This ten-fold difference between slow regeneration of brain AChE and rapid resynthesis of peripheral enzyme together with high brain selectivity of these compounds favors therapeutic effect over occurrence of side effects. This mechanism explains the low toxicity of metrifonate even at high (80–90%) AChE inhibition as compared to other ChEIs (*table II*).

4. Comparison of pharmacokinetic properties of cholinesterase inhibitors

A comparison of pharmacokinetic properties of five ChEI used in Alzheimer therapy is presented in *table IV*. Several important differences are apparent

with regard to metabolism as well as other characteristics. While tacrine, galanthamine and donepezil are metabolized through the hepatic route (P-450), rivastigmine and metrifonate are not. Metrifonate, in particular, is transformed non-enzymatically into the active compound which is a potent inhibitor of ChE. This metabolic difference is clinically important since elderly patients show a decrease of hepatic metabolism.

Another important characteristic is the difference in drug elimination reaching a long $T_{1/2}$ (73 h for donepezil) and a short $T_{1/2}$ (2 h for metrifonate). Bioavailability is maximal for galanthamine and metrifonate (100% and 90% respectively). Plasma protein binding is lowest (10 and 20% respectively) for galanthamine and metrifonate and highest (96%) for donepezil. Since elderly patients are generally treated with several drugs simultaneously, this is of particular interest in relation to drug interactions.

References

- [1] Anand R., Hartman R.D., Hayes P.E., An overview of the development of SDZ ENA 713, a brain selective cholinesterase inhibitor, in: Becker R., Giacobini E. (Eds.), *Alzheimer Disease: From molecular biology to therapy*, Birkhäuser, Boston, 1996, pp. 239–243.
- [2] Becker E., Giacobini E., Mechanisms of cholinesterase inhibition in senile dementia of the Alzheimer type, *Drug Dev. Res.* 12 (1988) 163–195.
- [3] Becker R., Giacobini E., Pharmacokinetics and pharmacodynamics of acetylcholinesterase inhibition, *Drug Dev. Res.* 14 (1988) 235–246.
- [4] Becker R., Colliver J., Elbe R., Effects of metrifonate, a long-acting cholinesterase inhibitor, *Drug Dev. Res.* 19 (1990) 425–434.
- [5] Becker R., Colliver J.A., Markwell S.J., Moricarty P., Unni L.K., Vicari S., Double-blind, placebo-controlled study of metrifonate, an acetylcholinesterase inhibitor, for Alzheimer disease. *Alz. Dis. Assoc. Dis.* 1 (1996) 124–131.
- [6] Becker R., Moricarty P., Unni L., Vicari S., Cholinesterase inhibitors as therapy in Alzheimer's disease: benefit to risk considerations in clinical application, in: Becker R., Giacobini E. (Eds.), *Alzheimer Disease: From molecular biology to therapy*, Birkhäuser, Boston, 1996, pp. 239–243.

- bini, E. (Eds.), *Alzheimer Disease: From molecular biology to therapy*, Birkhäuser, Boston, 1996, pp. 257–266.
- [7] Canal L., Imbimbo B.P., Clinical trials and therapeutics: Relationship between pharmacodynamic activity and cognitive effects of eptastigmine in patients with Alzheimer's disease, *Clin. Pharm. Ther.* 15 (1996) 49–59.
- [8] Cummings M.D., Cyrus P.A., Bieber F., Mas J., Orazem J., Gulanski B., Metrifonate treatment of the cognitive deficits in Alzheimer's disease, *Neurology* (1998) in press.
- [9] Dale H.H., The action of certain esters and ethers of choline, and their relation to muscarine, *J. Pharmacol.* 6 (1914) 147–190.
- [10] Davis K.L., Mohs R.C., Enhancement of memory by physostigmine, *N. Eng. J. Med.* 301 (1979) 946–956.
- [11] Doody R.S., Treatment of Alzheimer's disease, *Neurologist* 3 (1997) 279–289.
- [12] Farlow M., Gracon S.I., Hershey L.A., Lewis K.W., Sadowski C.H., Dolan-Ureno J.A., Controlled trial of tacrine in Alzheimer's disease, *J. Am. Med. Assoc.* 268 (1992) 2523–2529.
- [13] Giacobini E., Becker R., McIlhany M., Kumar V., Intracerebroventricular administration of cholinergic drugs: preclinical trials and clinical experience in Alzheimer patients, in: Giacobini E., Becker R., (Eds.), *Current Research in Alzheimer Therapy*, Taylor and Francis, New York, 1988, pp. 113–122.
- [14] Giacobini E., Cuadra G., Second and third generation cholinesterase inhibitors: From preclinical studies to clinical efficacy, in: Giacobini E., Becker R. (Eds.), *Alzheimer Disease: Therapeutic Strategies*, Birkhäuser, Boston, 1994, pp. 155–171.
- [15] Giacobini E., DeSarno P., Clark B., McIlhany M., The cholinergic receptor system of the human brain. Neurochemical and pharmacological aspects in aging and Alzheimer, in: Nordberg A., Fuxe K., Holmstedt B. (Eds.), *Progress in Brain Research*, Elsevier, Amsterdam, 1989, pp. 335–343.
- [16] Giacobini E., Cholinesterase inhibitors do more than inhibit cholinesterase, in: Becker R., Giacobini E. (Eds.), *Alzheimer Disease: From molecular biology to therapy*, Birkhäuser, Boston, 1996, pp. 187–204.
- [17] Giacobini E., Cholinomimetic therapy of Alzheimer disease: does it slow down deterioration?, in: Racagni G., Brunello N., Langer S.Z. (Eds.), *Recent Advances in the Treatment of Neurodegenerative Disorders and Cognitive Dysfunction*, International Academy of Biomedical Drug Research, Karger, New York, 1994, pp. 51–57.
- [18] Giacobini E., Cholinesterase inhibitors: From preclinical studies to clinical efficacy in Alzheimer disease, in: Quinn D., Balasubramaniam A.S., Doctor B.P., Taylor P. (Eds.), *Enzymes of the cholinesterase family*, Plenum Press, New York, 1995, pp. 463–469.
- [19] Imbimbo B.P., Lucchelli P.E., A pharmacodynamic strategy to optimize the clinical response to eptastigmine, in: Becker R., Giacobini E. (Eds.), *Alzheimer Disease: Therapeutic Strategies*, Birkhäuser, Boston, 1994, pp. 223–230.
- [20] Imbimbo B.P., Eptastigmine: A cholinergic approach to the treatment of Alzheimer's disease, in: Becker R., Giacobini E. (Eds.), *Alzheimer Disease: From molecular biology to therapy*, Birkhäuser, Boston, 1996, pp. 223–230.
- [21] Knapp M.J., Knopman D.S., Solomon P.R., A 30 week randomized controlled trial of high-dose tacrine in patients with Alzheimer's disease, *J. Am. Med. Assoc.* 271 (1994) 985–991.
- [22] Mattio T., McIlhany M., Giacobini E., Hallak M., The effects of physostigmine on acetylcholinesterase activity of CSF, plasma and brain, *Neuropharmacology* 25 (1986) 1167–1177.
- [23] Mc Keith I., Dubois B., Collins O., Meulien D., Efficacy and safety of metrifonate in Alzheimer's disease, Fifth Int. Geneva/Springfield Symposium on Advances in Alzheimer Therapy, Geneva (Abstr.), 1998, p. 74.
- [24] Mori F., Lai C.C., Fusi F., Giacobini E., Cholinesterase inhibitors increase secretion of APPs in rat brain cortex, *NeuroRep* 6 (1995) 633–636.
- [25] Morris J., Cyrus P., Orazem J., Mas J., Bieber F., Gulanski B., Metrifonate: potential therapy for Alzheimer's Disease, *Am. Soc. Neurol. Meeting*, Boston, Abstr. 155, 1997.
- [26] Moss D.E., Kobayashi H., Pacheco G., Palacios R., Perez R.G., Methanesulfonyl fluoride: a CNS selective cholinesterase inhibitor, in: Giacobini E., Becker R. (Eds.), *Current research in Alzheimer therapy*, Taylor and Francis, New York, 1988, 305–314.
- [27] Pettigrew L.C., Bieber F., Lettieri J., Wermeling D.P. et al., A study of the pharmacokinetics, pharmacodynamics and safety of metrifonate in Alzheimer's disease patients, *J. Clin. Pharmacol.* (1998), in press.
- [28] Rainer M., Mark T.H., Haushofer A., Galanthamine hydrobromide in the treatment of senile dementia of Alzheimer's type, in: Kewitz R., Thomsen A., Bickel C. (Eds.), *Pharmacological Interventions on Central Mechanisms in Senile Dementia*, W. Zuckschwerdt Verlag, München, 1989.
- [29] Rogers S.L., Friedhoff T., The efficacy and safety of donepezil in patients with Alzheimer's disease: Results of a US multicentre, randomized, double-blind, placebo-controlled trial, *Dementia* 7 (1996) 293–330.
- [30] Rogers S.L., Farlow M., Doody S.R. et al., A 24 week, double blind placebo controlled trial of donepezil in patients with AD, *Neurology* 50 (1998) 136–145.
- [31] Rupniak N.M.J., Field M.J., Samson N.A., Steventon M.J., Iversen S.D., Direct comparison of cognitive facilitation by physostigmine and tetrahydroaminoacridine in two primate models, *Neurobiol. Aging* 11 (1990) 609–613.
- [32] Summer W.K., Viesselman J.O., Marsh G.M., Candelora K., Use of THA in Treatment of Alzheimer-like dementia: pilot study in twelve patients, *Biol. Psychol.* 161 (1983) 45–153.
- [33] Thal J., Fuld P.A., Memory enhancement with oral physostigmine in Alzheimer's disease, *N. Eng. J. Med.* 308 (1983) 708–718.
- [34] Thal L., Fuld P.A., Masur D.M., Sharpless N.S., Oral physostigmine and lecithin improve memory in Alzheimer disease, *Ann. Neurol.* 13 (1983) 491–496.
- [35] Wilkinson D., Fulton B., Benfield P., Galanthamine, *Drugs Aging* 1 (1996) 60–66.
- [36] Wilkinson D., Galanthamine hydrobromide. Results of a group study, *Eight Congress Int. Psychoger.* Jerusalem, Abstr. 70, 1997.

The role of A β 42 in Alzheimer's disease

Steven G. Younkin

Mayo Clinic Jacksonville, Jacksonville, Florida 32224, USA

Abstract — Our recent studies of plasma, fibroblasts, transfected cells and transgenic mice show that a fundamental effect of the mutations linked to familial Alzheimer's disease (FAD) is to increase the extracellular concentration of A β 42. This effect of the FAD-linked mutations is likely to be directly related to the pathogenesis of Alzheimer's disease (AD) because A β 42 is deposited early and selectively in the senile plaques that are an invariant feature of all forms of AD. Thus our results provide strong evidence that the FAD-linked mutations all cause AD by increasing the extracellular concentration of A β 42 (43), thereby fostering A β deposition, and they support the hypothesis that cerebral A β deposition is an essential early event in the pathogenesis of all forms of AD. Interactions between the basal forebrain cholinergic system and A β that could influence AD pathogenesis are discussed. (©Elsevier, Paris)

Résumé — Rôle de l'A β 42 dans la maladie d'Alzheimer. Des études récentes effectuées sur le plasma, des fibroblastes, des cellules transfectées et des souris transgéniques montrent que les mutations liées à la maladie d'Alzheimer familial (FAD) augmentent la concentration extracellulaire d'A β 42. Cet effet des mutations liées à la FAD est probablement directement lié à la pathogénicité de la maladie d'Alzheimer puisque A β 42 est présent plus tôt et plus sélectivement dans les plaques séniles par rapport aux autres formes d'AD. Ces résultats confortent l'hypothèse que le dépôt d'A β est un événement essentiel dans toutes les formes d'AD. (©Elsevier, Paris)

amyloid / senile plaques / cholinergic system / familial Alzheimer's disease linked mutations

1. Amyloid β protein deposition in Alzheimer's disease

In patients with Alzheimer's disease (AD), large numbers of senile plaques are found throughout the cerebral neocortex and hippocampus. These senile plaques, which are present in small numbers in the brains of aged mammals and normal elderly individuals, are observed in large numbers only in AD and thus are specific for this disorder. 'Classic' neuritic senile plaques consist of a spherical cluster of altered neurites surrounding an amyloid core composed of 5–10 nm wide fibrils that can be visualized on light microscopy by staining with Congo Red or thioflavin S [30]. In these plaques, microglia are intimately associated with the amyloid cores, and there are surrounding astrocytes with processes that project through the altered neurites toward the amyloid core. In many cases of AD, amyloid fibrils are also found in the walls of cerebral blood vessels [10].

The principal proteinaceous component of the amyloid deposited in AD is an ~4 kDa peptide, referred to as the amyloid β protein (A β), that has been isolated both from plaque cores and meningeal vessels of AD brain [11, 17, 20, 21, 23]. Immunocytochemical studies with antisera to A β have established that A β is deposited not only in the neuritic plaques described above but also in large numbers of diffuse plaques, which are poorly circumscribed, immunoreactive lesions that are distinguished from neu-

ritic plaques in that they show minimal neuritic change or glial reaction [19, 32].

A β is produced by a single copy gene on chromosome 21, where it is encoded as an internal peptide within a large precursor protein referred to as the amyloid β protein precursor (β APP). In each of the various A β -containing β APP isoforms produced through alternative splicing of the β APP gene, the 42 amino acid A β peptide begins 99 residues from the carboxyl terminus of the β APP and it extends from the extracellular/intraluminal region (~28 amino acids) into the single membrane spanning domain (~14 amino acids) of the β APP [9, 17]. In 1992, my group and several others showed that normal processing of the β APP results in secretion of 4 kDa A β [13, 26, 28] that is readily detected in human cerebrospinal fluid, plasma, and medium conditioned by cultured cells. This soluble, secreted A β is primarily A β 1–40 (~90%), but minor amounts of A β 1–42 (~5–10%) and other A β species are also secreted [7, 29, 31].

Secreted A β is produced through the action of proteolytic activities referred to as β and γ secretase. Cleavage of full length β APP by β secretase produces a large secreted derivative, sAPP β , and an ~12 kDa cell-associated, C-terminal fragment with A β at its amino end. This ~12 kDa C-terminal fragment is then cleaved on the carboxyl side of A β by γ secretase to release A β . A third proteolytic activity, α secretase, cleaves full length β APP between A β 16

and A β 17 producing a large secreted derivative ending at A β 16, sAPP α , and an ~ 9 kDa cell-associated, C-terminal fragment that begins at A β 17.

Subsequent cleavage of this ~ 9 kDa derivative by γ secretase produces p3, a small, secreted C-terminal fragment of A β that begins at A β 17. Because it precludes production of full length A β , cleavage of the β APP holoprotein by α secretase is generally regarded as anti amyloidogenic.

Over the past decade, strong evidence has been obtained that the deposition of A β in senile plaques plays a seminal role in AD pathogenesis. Several lines of investigation indicate that the cholinergic system may influence A β deposition in ways that could significantly affect AD pathogenesis. First, there is excellent evidence that the activation of M1 and M3 receptors by acetylcholine can enhance cleavage of β APP by α secretase, an effect that appears to be mediated at least in part by activation of protein kinase C. Since this will tend to favor p3 production and reduce A β production, it could reduce extracellular A β concentration thereby slowing A β deposition. Thus, in principal, treatment of AD with anti-cholinesterases could enhance activation of M1 and M3 receptors thereby reducing A β deposition. In addition, the loss of basal forebrain cholinergic neurons that occurs as a prominent feature of AD, could, by reducing cholinergic transmission, set up positive feedback that tends to foster A β deposition increasingly as cholinergic neurons are lost. Second, Inestrosa and his colleagues have presented evidence that acetylcholinesterase interacts with A β , at least in vitro, in a way that tends to foster the formation of amyloid fibrils. The purpose of this brief review, which is excerpted in large part from a recent book chapter by the author, is to present the emerging evidence that A β deposition plays a seminal role in AD so that the potential importance of the interactions between A β and the cholinergic system (see other reports) is clear.

2. Effect of mutations that cause early onset familial Alzheimer's disease

It is now well established that early onset familial AD (FAD) can be caused by dominant mutations in the amyloid β protein precursor gene (APP) on chromosome 21[3, 12, 22], the presenilin 1 gene (PS1) on chromosome 14 [27], and the presenilin 2 gene (PS2) on chromosome 1 [18, 24]. If A β deposition is an essential early event in the pathologic process that causes AD, then each of the genetic changes known to cause AD must foster A β deposition. Thus one good way to test the A β deposition hypothesis is to determine if all the FAD linked mutations do,

in fact, cause changes that foster A β deposition. There are many ways that these mutations might cause A β deposition. Our laboratory and several others have examined the hypothesis that the FAD-linked mutations act by increasing the extracellular concentration of A β .

To evaluate the APP mutations known to cause FAD, we and others examined fibroblasts [5] from subjects carrying these mutations or transfected cells expressing the FAD-linked β APP mutations [2, 4, 29]. These studies established that the FAD-linked mutations on the amino (β APP 670N/671L) and carboxyl (β APP 717I, F, or G) sides of A β do, in fact, alter β APP processing in a way that fosters amyloid deposition either by coordinately increasing the extracellular concentration of A β 1–40 and A β 1–42 (43) (β APP 670N/671L) [2, 4, 29] or by selectively increasing the extracellular concentration of A β 1–42 (43) (β APP 717 mutations) [29], a peptide that forms insoluble amyloid fibrils more rapidly than A β 1–40 in vitro [16].

To determine if the FAD-linked APP mutations increase the extracellular concentration of A β in vivo and to assess the PS1 and PS2 mutations, we performed blinded comparisons of plasma A β 1–40 and A β 1–42 from carriers and controls [25]. Two studies were performed, the first compared 12 carriers with 31 non-carriers from the Swedish APPK670N,M671L kindred, and the second compared nine subjects with PS1G209V, PS1M146V, PS1H163R, or PS1E120D mutations; three subjects with PS2N141I mutations; and one subject with an APPV717I mutation with 14 controls. In addition, we analyzed plasma A β in 71 elderly patients with sporadic AD and 75 controls well matched for age, sex, and ethnicity. Remarkably, A β 1–42 (43) was significantly increased in essentially identical fashion in the plasma of subjects with each type of mutated gene known to cause early onset familial AD. In the 12 subjects with PS1/2 mutations, there was an unequivocal selective increase in the mean concentration of A β 1–42 (43). As expected from previous studies, there was a coordinate increase in A β 1–40 and A β 1–42 (43) in subjects with APPK670N,M671L mutations but a selective increase in A β 1–42 (43) in the subject with an APPV717I mutation. Plasma A β 42 (43) was increased in all of the presymptomatic carriers that were examined and it was not increased in the vast majority of symptomatic sporadic AD subjects that we examined. Thus, the elevated A β 42 (43) observed in subjects with FAD-linked mutations is not a secondary phenomenon of the AD state.

To establish that PS mutants increase brain A β , we analyzed the brains of transgenic mice expressing mutant (PS1M146L or PS1M146V) or wild type PS1 [8]. This analysis showed unequivocally that overex-

pression of mutant PS1 selectively increases the endogenous A β 42 in mouse brain. Thus, it established that these mutations increase A β 42 not only in plasma and peripheral cells but also in brain, the target organ for AD pathology. In addition, this study showed that PS1 mutations act in a truly dominant fashion because the normal endogenous PS1 genes present in the mice that were expressing mutant PS1 did not prevent the increase in A β 42. In another study carried out collaboratively with D. Borchelt, G. Thinakaran, S. Sisodia and their colleagues at Johns Hopkins University [1], we analyzed the brains of transgenic mice co-expressing β APP K670N,M671L and either wild type PS1 or mutant PS1A246E. In this experiment, the PS1A246E mutation significantly increased the relative amount of brain A β 42, even though the amount of total brain A β was substantially increased by the overexpression of the APP transgene. In this same study, N2a cells co-expressing human β APP and either wild type or mutant PS1 (PS1M146L, PS1A246E, PS1 Δ E9) were examined. Again, each of the mutations increased the relative amount of A42 found in the medium. In their study of transfected cells and transgenic mice expressing wild type or mutant presenilins, similar results were obtained by Citron et al. [6].

3. Discussion

Collectively, these studies of plasma, fibroblasts, transfected cells and transgenic mice show that a fundamental, generalized effect of the FAD-linked APP, PS1 and PS2 mutations is to increase the extracellular concentration of A β 42 (43). The plasma data are particularly important because they establish that these mutations increase extracellular A β 42 (43) in vivo. This effect of the FAD-linked mutations is likely to be directly related to the pathogenesis of AD because A β 42 (43) is deposited early and selectively in the senile plaques that are an invariant feature of all forms of AD [14, 15]. Thus our results provide strong evidence that the FAD-linked mutations all cause AD by increasing the extracellular concentration of A β 42 (43), thereby fostering A β deposition, and they support the hypothesis that cerebral A β deposition is an essential early event in the pathogenesis of all forms of AD. Other observations that provide additional support for a critical role for A β deposition in AD include: i) the finding that aggregated synthetic A β is toxic to cultured neurons in vitro; ii) the observation that A β can trigger the classic complement cascade in vitro; and iii) the finding that the A β deposited in neuritic plaques is intimately associated with proteins of the classic

complement cascade and with reactive microglia likely to be releasing cytokines and reactive free radicals that could have neurotoxic effects.

Based on these observations, it is reasonable to propose that extracellular A β deposition initiates a pathologic cascade that involves the formation of senile plaques, neurofibrillary tangles, and ultimately neuron and/or synapse loss resulting in dementia. The available data are consistent with this model and strongly support it. Thus it is important to develop drugs that lower A β 42 or that prevent the aggregation and deposition of A β in other ways, as there is an excellent chance that such drugs will provide effective therapy for AD, if compounds can be identified that enter the brain in sufficient concentration to be effective without causing unacceptable toxicity. As mentioned above, one way to do this may be by modulating effects that the basal forebrain cholinergic system has on A β metabolism.

References

- [1] Borchelt D.R., Thinakaran G., Eckman C.B., Lee M.K., Davenport F., Ratovitsky T., Prada C.M., Kim G., Seekins S., Yager D., Slunt H.H., Wang R., Seeger M., Levey A.I., Gandy S.E., Copeland N.G., Jenkins N.A., Price D.L., Younkin S.G., Sisodia S.S., Familial Alzheimer's disease-linked presenilin 1 variants elevate A β 1-42/1-40 ratio in vitro and in vivo, *Neuron* 17 (1996) 1005-1013.
- [2] Cai, X.D., Golde, T.E., Younkin S.G., Release of excess amyloid beta protein from a mutant amyloid beta protein precursor [see comments], *Science* 259 (1993) 514-516.
- [3] Chartier-Harlin M.C., Crawford F., Houlden, H., Warren A., Hughes D., Fidani L., Goate A., Rossor M., Roques P., Hardy J., Early-onset Alzheimer's disease caused by mutations at codon 717 of the beta-amyloid precursor protein gene, *Nature* 353 (1991) 844-846.
- [4] Citron M., Ottersdorf T., Haass C., McConlogue L., Hung A.Y., Seubert P., Vigo-Pelfrey C., Lieberburg I., Selkoe D.J., Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production, *Nature* 360 (1992) 672-674.
- [5] Citron M., Vigo-Pelfrey C., Teplow D.B., Miller C., Schenk D., Johnston J., Winblad B., Venizelos N., Lannfelt L., Selkoe D.J., Excessive production of amyloid beta-protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation, *Proc. Natl. Acad. Sci. USA* 91 (1994) 11993-11997.
- [6] Citron M., Westaway D., Xia W., Carlson G., Diehl T., Levesque G., Johnson-Wood K., Lee M., Seubert P., Davis A., Kholodenko D., Motter R., Sherrington R., Perry B., Yao H., Strome R., Lieberburg I., Rommens J., Kim S., Schenk D., Fraser P., St George Hyslop P., Selkoe D.J., Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice [see comments], *Nature Med.* 91 (1997) 67-72.
- [7] Dovey H.F., Suomensaaari-Chrysler S., Lieberburg I., Sinha S., Keim P.S., Cells with a familial Alzheimer's disease mutation produce authentic beta-peptide, *Neuroreport* 4 (1993) 1039-1042.

- [8] Duff K., Eckman C., Zehr C., Yu X., Prada C.M., Perez-Tur J., Hutton M., Buee L., Harigaya Y., Yager D., Morgan D., Gordon M.N., Holcomb L., Refolo L., Zenk B., Hardy J., Younkin S., Increased amyloid-beta42 (43) in brains of mice expressing mutant presenilin 1, *Nature* 383 (1996) 710-713.
- [9] Dyrks T., Weidemann A., Multhaup G., Salbaum J.M., Lemaire H.G., Kang J., Muller-Hill B., Masters C.L., Beyreuther K., Identification, transmembrane orientation and biogenesis of the amyloid A4 precursor of Alzheimer's disease, *EMBO J.* 7 (1988) 949-957.
- [10] Glenner G.G. in: Katzman R. (Ed.), *Banbury Report 15: Biological aspects of Alzheimer's disease*, Cold Spring Harbor Laboratory, New York, 1983, pp. 137-144.
- [11] Glenner G.G., Wong C.W., Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein, *Biochem. Biophys. Res. Commun.* 120 (1984) 885-890.
- [12] Goate A., Chartier-Harlin M.C., Mullan M., Brown J., Crawford F., Fidani L., Giuffra L., Haynes A., Irving N., James L., Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease [see comments], *Nature* 349 (1991) 704-706.
- [13] Haass C., Schlossmacher M.G., Hung A.Y., Vigo-Pelfrey C., Mellon A., Ostaszewski B.L., Lieberburg I., Koo E.H., Schenk D., Teplow D.B., Selkoe D.J., Amyloid B peptide is produced by cultured cells during normal metabolism, *Nature* 359 (1992) 322-325.
- [14] Iwatsubo T., Mann D.M., Odaka A., Suzuki N., Ihara Y., Amyloid beta protein (A beta) deposition: A beta 42 (43) precedes A beta 40 in Down syndrome [see comments], *Ann. Neurol.* 37 (1995) 294-299.
- [15] Iwatsubo T., Odaka A., Suzuki N., Mizusawa H., Nukina N., Ihara Y., Visualization of A beta 42 (43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42 (43), *Neuron* 13 (1994) 45-53.
- [16] Jarrett J.T., Berger E.P., Lansbury P.T., Jr., The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease, *Biochemistry* 32 (1993) 4693-4697.
- [17] Kang J., Lemaire H.G., Unterbeck A., Salbaum J.M., Masters C.L., Grzeschik K.H., Multhaup G., Beyreuther K., Muller-Hill B., The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor, *Nature* 325 (1987) 733-736.
- [18] Levy-Lahad E., Wasco W., Poorkaj P., Romano D.M., Oshima J., Pettingell W.H., Yu C.E., Jondro P.D., Schmidt S.D., Wang K., Candidate gene for the chromosome 1 familial Alzheimer's disease locus [see comments], *Science* 269 (1995) 973-977.
- [19] Masliah E., Terry R.D., Mallory M., Alford M., Hansen L.A., Diffuse plaques do not accentuate synapse loss in Alzheimer's disease, *Am. J. Pathol.* 137 (1990) 1293-1297.
- [20] Masters C.L., Simms G., Weinman N.A., Multhaup G., McDonald B.L., Beyreuther K., Amyloid plaque core protein in Alzheimer disease and Down syndrome, *Proc. Natl. Acad. Sci. USA* 82 (1985) 4245-4249.
- [21] Mori H., Takio K., Ogawara M., Selkoe D.J., Mass spectrometry of purified amyloid beta protein in Alzheimer's disease, *J. Biol. Chem.* 267 (1992) 17082-17086.
- [22] Mullan M., Crawford F., Axelman K., Houlden H., Lilius L., Winblad B., Lannfelt L., A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid, *Nat. Genet.* 1 (1992) 345-347.
- [23] Prelli F., Castano E., Glenner G.G., Frangione B., Differences between vascular and plaque core amyloid in Alzheimer's disease, *J. Neurochem.* 51 (1988) 648-651.
- [24] Rogaev E., Sherrington R., Levesque G., Ikeda M., St. George-Hyslop P., Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene, *Nature* 376 (1995) 775-778.
- [25] Scheuner D., Eckman C., Jensen M., Song X., Citron M., Suzuki N., Bird T.D., Hardy J., Hutton M., Kukull W., Larson E., Levy-Lahad E., Viitanen M., Peskind E., Poorkaj P., Schellenberg G., Tanzi R., Wasco W., Lannfelt L., Selkoe D., Younkin S., Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease [see comments], *Nat. Med.* 2 (1996) 864-870.
- [26] Seubert P., Vigo-Pelfrey C., Esch F., Lee M., Dovey H., Davis D., Sinha S., Schlossmacher M., Whaley J., Swindlehurst C., McCormack R., Wolfert R., Selkoe D., Lieberburg I., Schenk D., Isolation and quantitation of soluble Alzheimer's B peptide from biological fluids, *Nature* 359 (1992) 325-327.
- [27] Sherrington R., Rogaev E.I., Liang Y., Rogaeva E.A., Levesque G., Ikeda M., Chi H., Lin C., Li G., Holman K., Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease [see comments], *Nature* 375 (1995) 754-760.
- [28] Shoji M., Golde T.E., Ghiso J., Cheung T.T., Estus S., Shaffer L.M., Cai X.D., McKay D.M., Tintner R., Frangione B., Younkin S.G., Production of the Alzheimer amyloid beta protein by normal proteolytic processing, *Science* 258 (1992) 126-129.
- [29] Suzuki N., Cheung T.T., Cai X.D., Odaka A., Otvos L., Jr., Eckman C., Golde T.E., Younkin S.G., An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants, *Science* 264 (1994) 1336-1340.
- [30] Terry R.D., in: Davis R.L., Robertson D.M. (Eds.), *Textbook of Neuropathology*, Williams & Wilkins, Baltimore, 1985, pp. 824-841.
- [31] Vigo-Pelfrey C., Lee D., Keim P., Lieberburg I., Schenk D.B., Characterization of beta-amyloid peptide from human cerebrospinal fluid, *J. Neurochem.* 61 (1993) 1965-1968.
- [32] Yamaguchi H., Hirai S., Morimatsu M., Shoji M., Harigaya Y., Diffuse type of senile plaques in the brains of Alzheimer-type dementia, *Acta Neuropathol. (Berl.)* 77 (1988) 113-119.

Some cholinergic themes related to Alzheimer's disease: Synaptology of the nucleus basalis, location of m2 receptors, interactions with amyloid metabolism, and perturbations of cortical plasticity

M.-Marsel Mesulam

Cognitive Neurology and Alzheimer's Disease Center, Departments of Neurology and Psychiatry and Behavioral Sciences, Northwestern University Medical School, 320 East Superior Street, 11-450, Chicago, Illinois 60611, USA

Abstract — Cholinergic neurons in the nucleus basalis of Meynert (nbM) receive cholinergic, GABAergic and monoaminergic synapses. Only few of these neurons display the sort of intense m2 immunoreactivity that would be expected if they were expressing m2 as their presynaptic autoreceptor. The depletion of cortical m2 in Alzheimer's disease (AD) appears to reflect the loss of presynaptic autoreceptors located on incoming axons from the nucleus basalis of Meynert (nbM) and also the loss of postsynaptic receptors located on a novel group of nitric oxide producing interstitial neurons in the cerebral cortex. The defect of cholinergic transmission in AD may enhance the neurotoxicity of amyloid β , leading to a vicious cycle which can potentially accelerate the pathological process. Because acetylcholine plays a critical role in regulating axonal growth and synaptic remodeling, the cholinergic loss in AD can perturb cortical plasticity so as to undermine the already fragile compensatory reserve of the aging cerebral cortex. (©Elsevier, Paris)

Résumé — Quelques aspects cholinergiques de la maladie d'Alzheimer : synaptologie du noyau de Meynert, localisation des récepteurs M₂, interactions avec le métabolisme de la protéine amyloïde, perturbations de la plasticité corticale. Les neurones cholinergiques du noyau basal de Meynert (nbM) reçoivent des synapses cholinergiques, GABAergiques et mono-aminergiques. Une petite fraction de ces neurones présente une forte immunoréactivité pour les récepteurs m2, telle qu'on l'attendrait pour des auto-récepteurs présynaptiques. La diminution des récepteurs m2 corticaux dans la maladie d'Alzheimer (AD) semble refléter la perte d'auto-récepteurs présynaptiques localisés sur des axones provenant du nbM et aussi la perte de récepteurs postsynaptiques localisés sur un nouveau groupe de neurones interstitiels du cortex, produisant NO. Le défaut de transmission cholinergique dans la maladie d'Alzheimer peut augmenter la neurotoxicité de l'amyloïde β , dans un cercle vicieux d'accélération du processus pathologique. Étant donné le rôle critique de l'acétylcholine pour la croissance axonale et pour le remodelage des synapses, la perte cholinergique dans l'AD peut perturber la plasticité corticale et compromettre ainsi la réserve déjà fragile de compensation fonctionnelle du cortex cérébral vieillissant. (©Elsevier, Paris)

Ch4 / nitric oxide / cerebral cortex / dementia / memory

1. Introduction

The connections of the cerebral cortex can be divided into three broad categories: 1) Local-circuit projections; 2) long-distance projections interconnecting remote cortical areas with each other and with subcortical structures such as the thalamus, striatum and claustrum; and 3) diffuse modulatory projections which arise from relatively small regions of the basal forebrain and brainstem, which bypass the thalamus, and which are widely distributed throughout the cerebral cortex. These diffuse projections include cholinergic inputs from the basal forebrain, histaminergic inputs from the hypothalamus, dopaminergic inputs from the ventral tegmental area-substantia nigra, serotonergic inputs from the brainstem raphe nuclei, and noradrenergic inputs from the nucleus locus coeruleus [34].

The cholinergic projection from the nucleus basalis of Meynert (nbM) to the cerebral cortex constitutes the most massive of these diffuse modulatory pathways. The nbM and its cholinergic projections

continue to attract a great deal of attention because of their importance to many aspects of brain physiology and because of their severe degeneration in Alzheimer's disease (AD). In this very selective review, four related themes will be addressed: the emerging chemical synaptology of the nbM, the compartmentalization of m2 receptors, the interactions between the cholinergic system and beta amyloid (A β) metabolism, and the role of acetylcholine (ACh) in cortical synaptic plasticity.

2. Chemical synaptology of the primate nbM

The nbM contains cholinergic neurons (also known as the Ch4 group) intermingled with non-cholinergic neurons, most of which are GABAergic. Some of these GABAergic neurons are interneurons, others project to the cerebral cortex and innervate cortical inhibitory interneurons [14, 18]. The cell bodies (perikarya) of the Ch4 neurons contain indented nuclei, prominent nucleoli and cytoplasm rich in or-

ganelles [20, 42, 51]. Synaptic input to the perikaryon and proximal dendrites is sparse but increases distally.

The nbM has a complex neurotransmitter circuitry, much of which is still in the process of being unraveled. The nbM in the monkey and rat projects to the entire cerebral cortex but receives substantial cortical projections predominantly, if not exclusively, from limbic and paralimbic regions of the brain, including the amygdala [35, 52]. The relative paucity of reciprocal feedback projections from the neocortex is a property shared by the other ascending diffuse modulatory projections to the cerebral cortex. This aspect of connectivity may be essential for allowing these modulatory projections to rapidly shift information processing states throughout the cerebral cortex without the intervention of local feedback loops and in a way that is responsive primarily to the demands of the limbic system and the internal milieu [33]. The cortical inputs into the nbM are mostly glutamatergic but can also be GABAergic. At least in the rat, projections from the amygdala synapse directly onto cholinergic Ch4 neurons [54].

Dissociated cell cultures of nbM neurons show that they are responsive to acetylcholine, neurotensin, substance P, and L-glutamate [13, 39]. In the monkey, electron microscopic analyses of tissue labeled for the concurrent demonstration of the cholinergic marker choline acetyltransferase (ChAT) and the GABAergic marker glutamic acid decarboxylase (GAD) showed the presence of GABAergic symmetrical synapses onto the dendrites of both cholinergic and non-cholinergic nbM neurons [47]. Some of these synapses may represent the local axonal collaterals of GABAergic neurons, others may have an extrinsic origin in the cerebral cortex, amygdala, or nucleus accumbens.

The nbM also receives cholinergic innervation. Electron microscopic investigations of ChAT-immunolabeled nbM tissue in the rat shows that the vast majority of cholinergic terminals contact non-cholinergic neurons [30]. In the monkey, we find that cholinergic synapses onto cholinergic Ch4 neurons are somewhat more frequent and that they take the form of large asymmetrical synapses [47]. The precise source of the cholinergic input to the nbM is unknown but could include collaterals from cholinergic neurons of the basal forebrain or ascending projections from the Ch5–Ch6 group of pontomesencephalic cholinergic nuclei [21].

The monoaminergic innervation of the nucleus basalis has been investigated using autoradiography and immunoreactivity for individual receptor subtypes. 5HT immunoreactivity as a marker of serotonergic pathways, dopamine beta hydroxylase (DBH) immunoreactivity as a marker for noradrenergic pa-

thways, and tyrosine hydroxylase (TH) immunoreactivity as a marker for dopaminergic pathways. Such experiments showed that the nucleus basalis contains receptor sites for 5HT, dopamine, and norepinephrine [56]. In the rat, projections from dopaminergic ventral tegmental neurons, serotonergic raphe neurons, and noradrenergic locus coeruleus neurons have been identified in the nbM and have been shown to make direct synaptic contact with cholinergic Ch4 neurons [16, 21, 53].

In the human, TH and DBH immunoreactivity has been described in the septal area [15]. Our observations with the light microscope in the human brain show intense, preterminal-like TH and DBH immunoreactive axonal profiles in the nbM [46]. Reliable serotonin immunoreactivity has been difficult to obtain in the human brain. In the monkey brain, we detected a dense plexus of serotonin-immunoreactive as well as TH and DBH-positive axons within the nucleus basalis [46]. Electron microscopic analyses of tissue prepared for the concurrent visualization of ChAT and TH in the monkey showed the presence of moderately asymmetric TH-positive (presumably dopaminergic) synapses onto the dendrites of cholinergic (ChAT-positive) as well as non-cholinergic nbM neurons [47].

These observations show that the nbM provides a site for extensive cholinergic-monoaminergic interactions. In fact, $\alpha 2$ noradrenergic receptors can enhance the cortical release of acetylcholine, and the influence of noradrenergic stimulation upon the activity of cortical neurons appears to be mediated, at least in part, through the effect of such stimulation on the cholinergic neurons of the nbM [11, 49]. According to preliminary investigations, the nbM continues to receive dopaminergic and noradrenergic inputs in AD, suggesting that monoaminergic substances could be used in AD patients to stimulate the residual Ch4 neurons and their projections to the cerebral cortex [46].

3. Location of m2 receptors in the nbM and cerebral cortex

The investigation of cholinergic receptors entered a very productive phase with the molecular identification and cloning of five subtypes (m1–m5) of muscarinic receptors [6, 7, 26, 43]. All five of these receptors are G protein coupled: m1, m3, and m5 preferentially activate phospholipase C whereas m2 and m4 inhibit adenylyl cyclase activity [7]. Immunocytochemical experiments and in situ hybridization studies show that the m1 receptor subtype is found in the majority of cortical neurons, a distribution which is consistent with its role as the major

postsynaptic cholinergic receptor of the cerebral cortex [8, 29].

The m2 receptors are much less numerous and tend to have a different distribution in the cerebral cortex [32]. In AD, the severe loss of cortical cholinergic innervation is accompanied by a depletion of m2 and nicotinic receptors but a relative stability of m1 receptors [31, 48]. It has been customary to assume that m2 is a presynaptic autoreceptor on all cholinergic axons that extend from the nbM to the cerebral cortex and that these axons provide the major source of cortical m2 receptors. The depletion of m2 was therefore thought to represent the automatic consequence of the cholinergic axonal loss in AD. Recent observations, however, indicate that this view needs to be modified for two reasons. First, the presynaptic cholinergic axons coming from the nbM do not seem to make a substantial contribution to the overall pool of m2 receptors in the cerebral cortex. Secondly, the cerebral cortex contains m2 expressing neurons, the degeneration of which could provide an alternative source of m2 loss in AD.

The evidence for the presence of m2 receptors in the nbM is extensive. In dissociated nbM neurons, patch clamp techniques reveal currents associated with nicotinic as well as m2 muscarinic receptors [19]. The m2 receptor also appears to be the dominant species of cholinergic receptor in the nucleus basalis [27, 29, 50]. However, this receptor subtype is expressed at immunohistochemically detectable levels by only a third of Ch4 neurons in the rat [28]. In the monkey and human nbM, only 10–25% of Ch4 neurons express intense m2 immunoreactivity [38, 44]. In fact, the experimental elimination of Ch4 neurons in the monkey does not appear to alter the density or distribution of m2 receptors in the cerebral cortex [36]. Although the possibility that immunohistochemically undetectable levels of m2 receptors may act as autoreceptors for a large number of Ch4 neurons cannot be ruled out, these results are not compatible with the traditional view which attributes the m2 depletion in AD predominantly, if not exclusively, to the loss of axons ascending from the nbM to the cerebral cortex.

Cortical m2 immunostaining is located mostly in the neuropil but also in some perikarya, suggesting that this receptor subtype may function both as a pre-synaptic autoreceptor and also as a post-synaptic perikaryal receptor [29]. The m2 receptor subtype can also be associated with non-cholinergic terminals, suggesting that it may additionally act as a presynaptic heteroreceptor through which ACh may modulate the release of other transmitters [37]. In the cerebral cortex of the monkey, m2 receptors are expressed by two different classes of neuronal perikarya: 1) intracortical neurons which are mostly py-

ramidal in shape; and 2) polymorphic interstitial neurons most of which are located at the junction of the cerebral cortex with the underlying U-fibers of the white matter [36, 45].

Some of the m2-expressing pyramidal neurons may become the targets of degenerative processes in AD and can thus contribute to the overall loss of m2 receptors in these patients. In the primate brain, the interstitial polymorphic m2 neurons become quite conspicuous. They are present in all cortical areas and display particularly interesting properties. Nearly all of these neurons in the human and monkey brains express acetylcholinesterase (AChE) and nitric oxide synthase (NOS) activity [45]. Their neuronal cytoplasm is rich in endoplasmic reticulum and the nucleoli are quite prominent. The proximal dendrites are sparsely spiny. In the human brain, these neurons have dendritic spans of up to 1250 microns. Their processes reach the overlying cerebral cortex where they seem to come in potential contact with neurons and blood vessels.

Nitric oxide (NO) has been implicated in the regulation of vasodilation and synaptic plasticity. These interstitial neurons may thus provide a cholinceptive relay through which m2-mediated cholinergic stimulation may influence the release of NO and therefore cortical blood flow and plasticity. Alzheimer's disease does not alter the number of the NOS and m2-immunoreactive neurons but leads to a severe loss of their processes in the cerebral cortex [25]. The loss of these processes may make an additional contribution to the depletion of m2 receptors. The m2 depletion in AD may thus reflect degenerative changes sustained predominantly by neurons in the cerebral cortex rather than by neurons in the nbM.

4. Cholinergic-A β interactions in AD

The possible linkage between the cholinergic depletion in AD and the metabolism of A β has attracted considerable interest. One group of investigators had reported that lesions in the nbM of rats induced the formation of plaque-like deposits in the cerebral cortex [1], and another that neurons of the nbM overexpressed amyloid precursor protein (APP) in a way that could promote the formation of plaques in AD [9]. Neither result has yet received widespread confirmation and there is currently little support for the hypothesis that A β plaques are 'caused' either by the degeneration or dysfunction of nbM neurons.

Cholinergic axons of the cerebral cortex are lost early in the course of AD at a time when many other neuronal systems remain relatively spared [17]. The biological mechanisms that regulate this selective vulnerability remain poorly understood. There are no

local correlations between the density of A β plaques and the severity of the cholinergic depletion [17]. Furthermore, experimental injections of A β in the rat cerebral cortex are no more toxic to cholinergic axons than to other types of axonal systems [12]. There is, thus, no support for the hypothesis that the selective vulnerability of cholinergic innervation reflects an unusual susceptibility to A β neurotoxicity.

Although cholinergic degeneration does not cause A β plaques, and A β plaques do not cause the preferential vulnerability of cortical cholinergic axons, complex interactions of potentially profound pathophysiological significance are being identified between cholinergic neurotransmission and A β metabolism. For example, m1- and m3-mediated muscarinic stimulation of cortical neurons promotes the processing of APP by the α secretase pathway [40]. This pathway splits APP in the middle of the A β domain and therefore precludes the subsequent release of insoluble A β . Additional *in vitro* experiments have shown that nicotine-mediated cholinergic neurotransmission may protect neurons from A β neurotoxicity [23]. These two sets of experiments imply that a cholinergic depletion may not only increase the deposition of A β plaques but also their local neurotoxic effects.

Numerous experiments have also shown that A β disrupts ACh synthesis and the signal transduction events associated with cholinergic neurotransmission [2, 22]. It appears, therefore, that AD may be associated with a vicious cycle wherein the cholinergic depletion intensifies both the production and neurotoxicity of A β which, in turn, further reduces the effectiveness of cholinergic neurotransmission (*figure 1*). Interfering with this vicious cycle through the use of m1, m3 and nicotinic agonists may have potentially beneficial effects in AD.

5. Acetylcholine and plasticity

The role of ACh in plasticity has been investigated for many years [4]. Time lapse photography in cultured neurons, for example, show that acetylcholine inhibits neurite outgrowth and that this effect

is mediated through nicotinic receptors [41]. Furthermore, the selective lesioning of cortical cholinergic innervation in the rat was shown to interfere with experience-dependent plasticity in the barrel fields. In one experiment, all whiskers except for D2 and D3 were trimmed. This led to an experience-dependent pairing between the D2 and D3 barrel fields in the cerebral cortex so that the D2 neurons started to show a greater responsivity to D3 than to the adjacent D1 which had been trimmed. This pairing, indicative of experience-induced synaptic plasticity, could not be obtained in rats with selective immunotoxic lesions of the cholinergic neurons in the nbM [3]. In another experiment on newborn rat pups, barrels representing intact whiskers failed to show the expected expansion into the territory of barrels representing trimmed whiskers in animals with nbM lesions [55]. Furthermore, pairing auditory stimuli with the electrical stimulation of the nbM in adult rats caused a long-lasting reorganization of primary auditory cortex so that the area optimally responsive to the paired tone increased substantially. This plasticity was not observed in rats with nbM lesions [24].

The impact of cholinergic neurotransmission has traditionally been attributed to its immediate effect on the ionic channels of cholinceptive neurons. The observations listed above suggest that acetylcholine may also have an equally important and much more prolonged impact as a promoter of synaptic plasticity. Synaptic plasticity is not confined to early development. It persists throughout adulthood, serving multiple purposes ranging from the experience-induced reorganization of synaptic circuits to the reactive synaptogenesis necessitated by physiological synapse turnover [5, 10]. The apparently pivotal role of acetylcholine in the regulation of neuroplasticity suggests that its depletion in AD may accelerate the pathological process by undermining the capacity for compensatory reorganization.

6. Overview

The initial hope, based on the relationship between dopamine and Parkinson's disease, that AD would turn out to be a cholinergic disease and that it would yield to cholinergic therapy has not materialized. There is currently no comprehensive 'cholinergic theory' of AD pathogenesis. However, there is a 'cholinergic lesion' in AD, with potentially vast implications for pathophysiology and pharmacotherapy. The depletion of cortical cholinergic innervation in AD is early and severe. This depletion may disrupt neural circuits involved in attention and memory, promote A β deposition and neurotoxicity, and

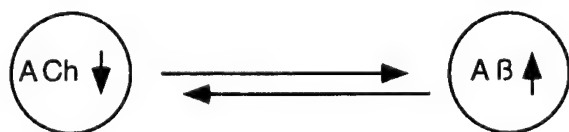


Figure 1. A schematic representation of the cholinergic 'vicious cycle' in AD. The cholinergic depletion promotes the deposition and toxicity of A β and A β decreases the effectiveness of cholinergic neurotransmission.

perturb neural plasticity. Each of these areas can become the target of specifically tailored cholinergic therapies.

Acknowledgments

Supported in part by NS 20285 and AG 13854.

References

- [1] Arendash G.W., Millard W.J., Dunn A.J., Meyer E.M., Long-term neuropathological and neurochemical effects of nucleus basalis lesions in the rat, *Science* 238 (1987) 952-956.
- [2] Auld D.S., Kar S., Quirion R., β -Amyloid peptides as direct cholinergic neuromodulators: a missing link?, *Trends Neurosci.* 21 (1998) 43-49.
- [3] Baskerville K.A., Schweitzer J.B., Herron P., Effects of cholinergic depletion on experience-dependent plasticity in the cortex of the rat, *Neuroscience* 80 (1997) 1159-1169.
- [4] Bear M.F., Singer W., Modulation of visual cortical plasticity by acetylcholine and noradrenaline, *Nature* 320 (1986) 172-176.
- [5] Black J.E., Polinsky M., Greenough W.T., Progressive failure of cerebral angiogenesis supporting neural plasticity in aging rats, *Neurobiol. Age* 10 (1989) 353-358.
- [6] Bonner T.I., Buckley N.J., Young A.C., Brann M.R., Identification of a family of muscarinic acetylcholine receptor genes, *Science* 237 (1987) 527-532.
- [7] Bonner T.I., Young A.C., Brann M.R., Buckley N.J., Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes, *Neuron* 1 (1988) 403-410.
- [8] Buckley N.J., Bonner T.I., Brann M.R., Localization of muscarinic receptor mRNAs in rat brain, *J. Neurosci.* 8 (1988) 4646-4652.
- [9] Cohen M.L., Golde T.E., Usiak M.F., Younkin L.H., Younkin S.G., In situ hybridization of nucleus basalis neurons shows increased β -amyloid mRNA in Alzheimer's disease, *Proc. Natl. Acad. Sci. USA* 85 (1988) 1227-1231.
- [10] Cotman C.W., Nieto-Sampedro M., Cell biology of synaptic plasticity, *Science* 225 (1984) 1287-1294.
- [11] Dringenberg H.C., Vanderwolf C.H., Neocortical activation: modulation by multiple pathways acting on central cholinergic and serotonergic systems, *Exp. Brain Res.* 116 (1997) 160-174.
- [12] Emre M., Geula C., Ransil B.J., Mesulam M.M., The acute neurotoxicity and effects upon cholinergic axons of intracerebrally injected beta-amyloid in the rat brain, *Neurobiol. Aging* 13 (1992) 553-559.
- [13] Farkas R.H., Nakajima S., Nakajima Y., Neurotensin excites basal forebrain cholinergic neurons: ionic and signal-transduction mechanisms, *Proc. Natl. Acad. Sci. USA* 91 (1994) 2853-2857.
- [14] Freund T.F., Meskenaite V., γ -Aminobutyric acid-containing basal forebrain neurons innervate inhibitory interneurons in the neocortex, *Proc. Natl. Acad. Sci. USA* 89 (1992) 738-742.
- [15] Gaspar P., Berger B., Alvarez C., Vigny A., Henry J.P., Catecholaminergic innervation of the septal area in man: immunocytochemical study using TH and DBH antibodies, *J. Comp. Neurol.* 241 (1985) 12-33.
- [16] Gaykema R.P., Zaborzsky L., Direct catecholaminergic-cholinergic interactions in the basal forebrain. II. Substantia nigra-ventral tegmental area projections to cholinergic neurons, *J. Comp. Neurol.* 374 (1996) 555-577.
- [17] Geula C., Mesulam M.-M.: Cholinergic systems and related neuropathological predilection patterns in Alzheimer disease. in: Terry R.D., Katzman R. and Bick K.L. (Eds.), *Alzheimer Disease*, Raven Press, New York, 1994, pp. 263-294.
- [18] Gritti I., Mainville L., Jones B.E., Codistribution of GABA with acetylcholine-synthesizing neurons in the basal forebrain of the rat, *J. Comp. Neurol.* 329 (1993) 438-457.
- [19] Harata N., Tateishi N., Akaike N., Acetylcholine receptors in dissociated nucleus basalis of Meynert neurons of the rat, *Neurosci. Lett.* 130 (1991) 153-156.
- [20] Ingham C.A., Bolam J.P., Wainer B.H., Smith A.D., A correlated light and electron microscopic study of identified cholinergic basal forebrain neurons that project to the cortex in the rat, *J. Comp. Neurol.* 239 (1985) 176-192.
- [21] Jones B., Cuello A.C., afferents to the basal forebrain cholinergic cell area from pontomesencephalic-catecholamine, serotonin and acetylcholine-neurons, *Neuroscience* 31 (1989) 37-61.
- [22] Kelly J.F., Furukawa K., Barger S.W., Rengen M.R., Mark R.J., Blanc E.M., Roth G.S., Mattson M.P., Amyloid β -peptide disrupts carbachol-induced muscarinic cholinergic signal transduction in cortical neurons, *Proc. Natl. Acad. Sci. USA* 93 (1996) 6753-6758.
- [23] Kihara T., Shimohama S., Sawaa H., Kimura J., Kume T., Kochiyama H., Maeda T., Akaike A., Nicotinic receptor stimulation protects neurons against β -amyloid toxicity, *Ann. Neurol.* 42 (1997) 159-163.
- [24] Kilgard M.P., Merzenich M.M., Cortical map reorganization enabled by nucleus basalis activity, *Science* 279 (1998) 1714-1718.
- [25] Kowall N.W., Beal M.F., Cortical somatostatin, neuropeptide Y, and NADPH diaphorase neurons: Normal anatomy and alterations in Alzheimer's disease, *Ann. Neurol.* 23 (1988) 105-114.
- [26] Kubo T., Fukuda K., Mikami A., Maeda A., Takahashi H., Mishina M., Haga T., Haga K., Ichiyama A., Kangawa K., Kojima M., Matsuo H., Hirose T., Numa S., Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor, *Nature* 323 (1986) 411-416.
- [27] Levey A.I., Edmunds S.M., Heilman C.J., Desmond T.J., Frey K.A., Localization of muscarinic m3 receptor protein and M3 receptor binding in the rat brain, *Neuroscience* 63 (1994) 207-221.
- [28] Levey A.I., Edmunds S.M., Hersch S.M., Wiley R.G., Heilman C.J., Light and electron microscopic study of m2 muscarinic acetylcholine receptor in the basal forebrain of the rat, *J. Comp. Neurol.* 351 (1995) 339-356.
- [29] Levey A.I., Kitt C.A., Simonds W.F., Price D.L., Brann M.R., Identification and localization of muscarinic acetylcholine receptor protein in brain with subtype-specific antibodies, *J. Neurosci.* 11 (1991) 3218-3226.
- [30] Martinez-Murillo R., Villalba R.M., Rodrigo J., Immunocytochemical localization of cholinergic terminals in the region of the nucleus basalis magnocellularis of the rat: A correlated light and electron microscopic study, *Neuroscience* 36 (1990) 361-376.
- [31] Mash D.C., Flynn D.D., Potter L.T., Loss of M2 muscarinic receptors in the cerebral cortex in Alzheimer's disease and experimental cholinergic denervation, *Science* 228 (1985) 115-117.

- [32] Mash D.C., White W.F., Mesulam M.M., Distribution of muscarinic receptor subtypes within architectonic subregions of the primate cerebral cortex, *J. Comp. Neurol.* 278 (1988) 265–274.
- [33] Mesulam M.-M., Asymmetry of neural feedback in the organization of behavioral states, *Science* 237 (1987) 537–538.
- [34] Mesulam M.-M., Large-scale neurocognitive networks and distributed processing for attention, language, and memory, *Ann. Neurol.* 28 (1990) 597–613.
- [35] Mesulam M.-M., Mufson E.J., Neural inputs into the nucleus basalis of the substantia innominata (Ch4) in the rhesus monkey, *Brain* 107 (1984) 253–274.
- [36] Mrzljak L., Levey A.I., Belcher S., Goldman-Rakic P.S., Localization of the m2 muscarinic acetylcholine receptor protein and mRNA in cortical neurons of the normal and cholinergically deafferented rhesus monkey, *J. Comp. Neurol.* 390 (1998) 112–132.
- [37] Mrzljak L., Levey A.I., Goldman-Rakic P.S., Association of m1 and m2 muscarinic receptor proteins with asymmetric synapses in the primate cerebral cortex: Morphological evidence for cholinergic modulation of excitatory neurotransmission, *Proc. Natl. Acad. Sci. USA* 90 (1993) 5194–5198.
- [38] Mufson E.J., Jaffar S., Levey A.I., m2 muscarinic acetylcholine receptor-immunoreactive neurons are not reduced within the nucleus basalis in Alzheimer's disease: relationship with cholinergic and galaninergic perikarya, *J. Comp. Neurol.* 392 (1998) 313–329.
- [39] Nakajima Y., Nakajima S., Obata K., Carlson C.G., Yamaguchi K., Dissociated cell culture of cholinergic neurons from nucleus basalis of Meynert and other basal forebrain nuclei, *Proc. Natl. Acad. Sci. USA* 82 (1985) 6325–6329.
- [40] Nitsch R.M., Slack B.E., Wurtman R.J., Growdon J.H., Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors, *Science* 258 (1992) 304–307.
- [41] Owen A., Bird M., Acetylcholine as a regulator of neurite outgrowth and motility in cultured embryonic mouse spinal cord, *NeuroReport* 6 (1995) 2269–2272.
- [42] Palacios G., The endomembrane system of cholinergic and non-cholinergic neurons in the medial septal nucleus and vertical limb of the diagonal band of Broca: A cytochemical and immunocytochemical study, *J. Histochem. Cytochem.* 38 (1990) 563–571.
- [43] Peralta E.G., Ashkenazi A., Winslow J.W., Smith D.H., Ramachandran J., Capon D.J., Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors, *EMBO J.* 6 (1987) 3923–3929.
- [44] Smiley J.F., Levey A.I., Mesulam M.-M., m2 muscarinic receptor immunolocalization in cholinergic cells of the monkey basal forebrain, *Soc. Neurosci. Abstr.* 23 (1997) 2021.
- [45] Smiley J.F., Levey A.I., Mesulam M.-M., Infracortical interstitial cells concurrently expressing m2-muscarinic receptors, AChE, and NADPH-d in the human and monkey cerebral cortex, *Neuroscience* 84 (1998) 755–769.
- [46] Smiley J.F., Mesulam M.-M., Dopamine, norepinephrine, and serotonin axons in the human and monkey nucleus basalis and in Alzheimer's disease, *Soc. Neurosci. Abstr.* 21 (1995) 1621.
- [47] Smiley J.F., Mesulam M.-M., Cholinergic neurons of the nucleus basalis of Meynert (Ch4) receive cholinergic, catecholaminergic, and GABAergic synapses: an electron microscopic investigation in the monkey, *Neuroscience* (1998), in press.
- [48] Svensson A.L., Warpman U., Hellstrom-Lindahl E., Bogdanovic N., Lannfelt L., Nordberg A., Nicotinic receptors, muscarinic receptors and choline acetyltransferase activity in the temporal cortex of Alzheimer patients with differing apolipoprotein E genotypes, *Neurosci. Lett.* 232 (1997) 37–40.
- [49] Tellez S., Colpaert F., Marien M., The α_2 -adrenoceptor antagonist, (+)-efaroxan, enhances acetylcholine release in the rat cortex in vivo, *Eur. J. Pharmacol.* 277 (1995) 113–116.
- [50] Vilaró M.T., Wiederhold K.-H., Palacios J.M., Mengod G., Muscarinic M2 receptor mRNA expression and receptor binding in cholinergic and non-cholinergic cells in the rat brain: a correlative study using in situ hybridization histochemistry and receptor autoradiography, *Neuroscience* 47 (1992) 367–393.
- [51] Walker L.C., Tigges M., Tigges J., Ultrastructure of neurons in the nucleus basalis of Meynert in squirrel monkey, *J. Comp. Neurol.* 217 (1983) 158–166.
- [52] Záborsky L., Gaykema R.P., Swanson D.J., Cullinan W.E., Cortical input to the basal forebrain, *Neuroscience* 79 (1997) 1051–1078.
- [53] Záborsky L., Cullinan W.E., Direct catecholaminergic-cholinergic interactions in the basal forebrain. I. Dopamine- β -hydroxylase- and tyrosine hydroxylase input to cholinergic neurons, *J. Comp. Neurol.* 374 (1996) 535–554.
- [54] Záborsky L., Leranthe C., Heimer L., Ultrastructural evidence of amygdalofugal axons terminating on cholinergic cells of the rostral forebrain, *Neurosci. Lett.* 52 (1984) 219–225.
- [55] Zhu X.O., Waite P.M.E., Cholinergic depletion reduces plasticity of barrel field cortex, *Cereb. Cortex* 8 (1998) 63–72.
- [56] Zilles K., Werner L., Qü M., Schleicher A., Gross G., Quantitative autoradiography of 11 different transmitter binding sites in the basal forebrain region of the rat-evidence of heterogeneity in distribution patterns, *Neuroscience* 42 (1991) 473–481.

The effects of the acetylcholinesterase inhibitor ENA713 and the M1 agonist AF150(S) on apolipoprotein E deficient mice

Shira Chapman^a, Abraham Fisher^b, Marta Weinstock^c, Rachel Brandies^b,
Esther Shohami^c, Daniel M. Michaelson^a

^a*Department of Neurobiochemistry, Tel-Aviv University, Tel-Aviv, Israel*

^b*Department of Pharmacology, Israel Institute for Biological Research, Ness-Ziona, Israel*

^c*Department of Pharmacology, The Hebrew University, Jerusalem, Israel*

Abstract — Apolipoprotein E (apoE)-deficient and control mice were treated chronically with either the acetylcholinesterase (AChE) inhibitor ENA713, or the M1 muscarinic agonist AF150(S). Both treatments reversed the spatial working memory impairment of apoE-deficient mice but they differed in their effects on the levels of brain AChE activity. AF150(S) enhanced the brain AChE activity of apoE-deficient mice and rendered it similar to that of the untreated controls, whereas ENA713 reduced the brain AChE activity of control mice but had no effect on that of apoE-deficient mice. These findings suggest that AChE inhibition and M1 muscarinic activation have similar beneficial cognitive effects on apoE-deficient mice, but that the cellular and molecular mechanisms underlying these effects differ. (©Elsevier, Paris)

Résumé — Effets de l'inhibiteur d'AChE, ENA 713 et de l'agoniste AF 150(S) du récepteur M1 chez les souris déficientes en apolipoprotéine E. Le traitement de souris déficientes en apo E par l'ENA 713 (inhibiteur d'AChE) ou le AF150(S), agoniste M1, améliorent la mémoire spatiale de ces souris mais par des mécanismes moléculaires différents. (©Elsevier, Paris)

apolipoprotein E / acetylcholinesterase / muscarinic animal model

1. Introduction

Genetic studies suggest that apolipoprotein E (apoE) genotype is a major risk factor for Alzheimer's disease (AD) [11]. Furthermore, neuropathological studies revealed that brain cholinergic deficits are more pronounced in AD patients which carry the allele E4 (apoE4), which is the risk factor for AD, than in patients which carry other apoE alleles. This led to the suggestion that apoE plays an important role in brain cholinergic function [10].

ApoE-deficient mice [9], provide a useful model system for studying the role of apoE in cholinergic and in other brain neuronal functions. Indeed, we have previously shown that apoE-deficient mice have memory impairments which are associated with synaptic loss of distinct neuronal pathways the magnitude of which is most pronounced in the basal forebrain cholinergic system [6]. The findings that apoE-deficient mice have impairments both in their cognitive function and cholinergic systems, renders them an attractive model system for examining the efficacy of distinct cholinergic therapeutic strategies. Centrally acting acetylcholinesterase (AChE) inhibitors [7] and M1-selective muscarinic agonists [4], have been reported to have some beneficial effect in AD patients.

In the present study we compared the effects of a prototype of each of these agents in apoE-deficient

and control mice. Two groups of mice were treated for 3 weeks with either the brain specific AChE inhibitor ENA713 [2, 16], which is the S-isomer of RA7, originally developed by Weinstock et al. [15] or with the M1 muscarinic agonist 1-methyl-piperidine-4-spiro(2'-methylthiazoline) (AF150(S)) [3] and by comparing the resulting cognitive and neurochemical effects compared.

2. Materials and methods

2.1. Behavioral studies

ApoE-deficient mice (4 months old, $n = 10$ in each group) and age-matched controls of the same parent line (C57BL/6J), provided by Dr. J.L. Breslow [9], were subjected to the Morris water maze-working memory test (delayed matching to sample) for 5 successive days as previously described [5]. The drugs (0.5 mg/kg AF150(S) p.o. and 1.5 mg/kg ENA713 s.c.) were administered daily for 1 week prior to the initiation of the behavioral experiment. Administration was continued during the 5 days of the behavioral test, in which AF150(S) was administered 1 h before testing and ENA713 3–4 h after testing. Results were analyzed for each mouse as the mean path length of the second trial, divided by the mean path length of the first trial, as previously described [5]. The ratios obtained for each mouse represent the relative gain in performance from the first to the second trial and are thus a measure of working memory function.

2.2. AChE histochemistry

Four to five of the ten mice of each group which were tested in the Morris water maze were randomly selected for histochemical evaluation of their brains. In order to enable a comparison to be made of the results of the present study to those of previous experiments [5], the mice were treated with the drugs for an additional week. The AF150(S) and ENA713 treated mice were then killed at 4 h and 20 h respectively following the last administration of the drug. Their brains were excised sectioned and processed as previously described [1] and 3–5 consecutive frozen sections of each brain area were stained histochemically for AChE activity utilizing the Karnovsky method [8]. The resulting intensities of AChE staining of the indicated brain areas were determined by computerized image analysis [1]. Results, from the stained sections of each mouse brain at the level of the septum and the hippocampus, are presented as the mean optical density score for each brain area of each mouse. The intensities of staining of the different mice groups were compared using a two-way ANOVA, followed by simple main effects contrast analysis.

3. Results

The effects of the AChE inhibitor ENA713 and of the M1 agonist AF150(S) on the working memory of apoE-deficient and control mice were determined utilizing the Morris water maze and a paradigm in which the extent of improvement in performance of the mice was monitored by measurements of the ratio between the second and first of two identical

daily trials. The results thus obtained are depicted in *figure 1*. As can be seen, the sham-treated apoE-deficient mice performed in both the AF150(S) and ENA713 experiments less well (i.e., path length ratios of 1.25 ± 0.34 and 1.25 ± 0.2 respectively), than the corresponding controls (i.e., path length ratios of 0.80 ± 0.18 and 0.82 ± 0.03 respectively). This is in accordance with previous reports [5, 6] and suggests that the working memory of apoE-deficient mice is impaired. Treatment of the two mice groups with either AF150(S) or ENA713 resulted in significant improvements of the performances of the apoE-deficient mice and rendered their working memory performance similar to those of control mice (*figure 1*). Accordingly, the path lengths ratios of the AF150(S) treated apoE-deficient and control mice were respectively 0.74 ± 0.1 and 0.78 ± 0.08 , and the corresponding ratios of the ENA713 treated apoE-deficient and control mice were 0.75 ± 0.07 and 0.89 ± 0.1 . Statistical analysis of this data revealed for both drugs a main effect of group (control versus apoE $P < 0.05$), and an interaction of group by treatment ($P < 0.05$).

The extent to which the AF150(S) and ENA713 mediated memory improvements are associated with neurochemical changes was monitored by histochemical measurements of brain AChE activity. As can be seen (*figure 2*) the two drugs had different effects on the hippocampal and cortical AChE activities of the two mice groups. Whereas AF150(S) reversed

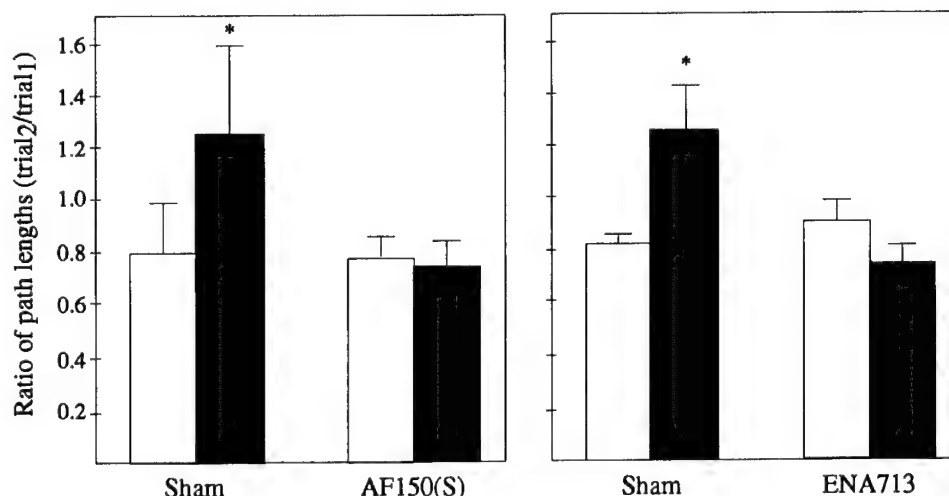


Figure 1. The effects of AF150(S) and ENA713 on the working memory of apoE-deficient and control mice. Spatial working memory was tested in the Morris water maze by measuring the ratio between the escape latency in the second trial to that of the first trial as described in *Materials and methods*. Results presented are the mean \pm SEM of 9–10 mice in each group. The black bars correspond to apoE-deficient mice whereas the white bars correspond to controls. The drugs were administered once a day for 1 week prior to the initiation of the behavioral testing and then during the experiment. Mice which did not receive the drug were sham-treated with PBS. * $P < 0.05$ relative to sham-treated control.

the AChE deficit of the apoE-deficient mice by elevating their AChE activities, ENA713 had no effect on the AChE activities of these mice. Furthermore, AF150(S) induced a small and non-significant decrease in the cortical and hippocampal AChE activities of the controls, whereas ENA713 caused marked decreases in the corresponding control AChE activities. Thus, AF150(S) abolished the difference in AChE activity between the two mice groups by enhancing the AChE activities of the apoE-deficient mice while having no effect on the corresponding control AChE activities (group by treatment $P < 0.001$). In contrast, ENA713 which also abolished the difference in AChE activities between the two mice groups, causes this effect by lowering the AChE activities of control mice while having no effect on

those of the apoE-deficient mice (group by treatment $P < 0.03$).

The extent of a correlation between the levels of either cortical or hippocampal AChE activity and so the ratio of path lengths of individual treated and untreated mice was investigated. The results obtained are depicted in figure 3. As can be seen the AF150(S) treated and non-treated apoE-deficient and control mice showed a significant correlation between the levels of AChE activity and the cognitive performance of the individual mice. The higher the AChE activity the better the working memory performance. This correlation was statistically significant with regards to both cortical AChE activities ($r = -0.717$, $P < 0.01$), and to hippocampal ($r = -0.558$, $P < 0.05$) AChE activities. In contrast, ENA713 had the opposite effect to that of AF150(S),

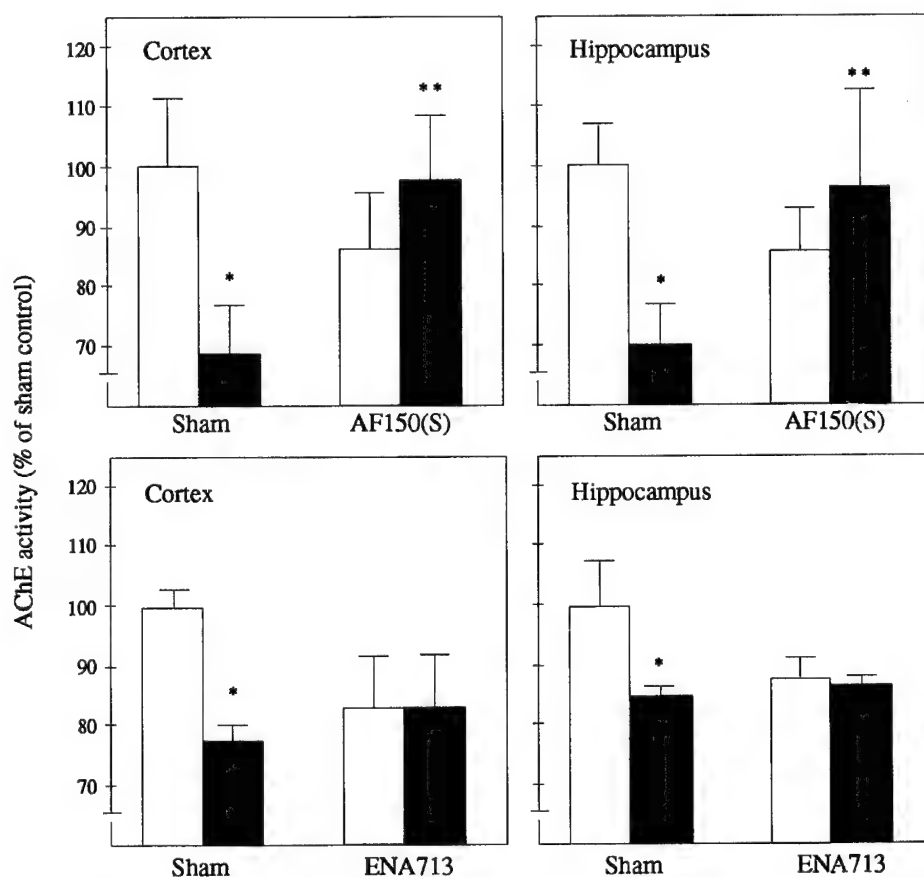


Figure 2. The effects of AF150(S) and ENA713 on AChE activities in the cortex and the hippocampus of apoE-deficient (dark) and control (white) mice. Mice were treated with the drugs for 3 weeks. The mice were killed 4 h following the last treatment with AF150(S) and 20 h following the last treatment with ENA713, after which their brains were processed and stained as described in *Materials and methods*. Stained sections of 4–5 mice in each group were scanned for each brain area and the intensities of the staining were averaged for each area of each mouse. Results presented are the means \pm S.E.M. of each group. * $P < 0.03$ relative to sham-treated control. ** $P < 0.05$ relative to sham-treated apoE-deficient mice.

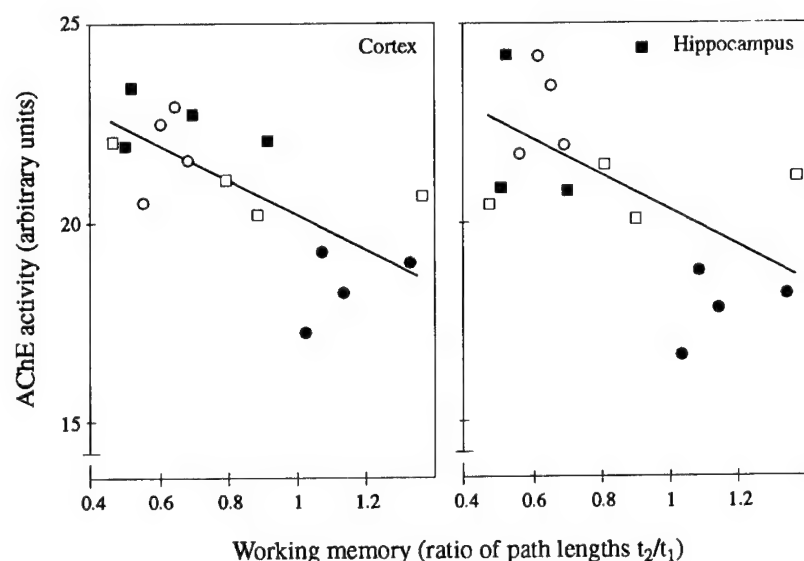


Figure 3. Correlation between working memory and AChE activities in the hippocampus and cortex of AF150(S) treated and non-treated apoE-deficient and control mice. Working memory and AChE activities were assessed as described in *Materials and methods*. Results presented are the individual values of each apoE-deficient mouse treated with AF150(S) (black squares) or sham treated (black circles) and of each control mouse treated with AF150(S) (white squares) or sham-treated (white circles). Correlation analysis of the results presented revealed $R = -0.717$ ($P < 0.01$) for the cortical AChE activity and $R = -0.558$ ($P < 0.05$) for the hippocampal AChE activity.

on AChE activity and thus there was no significant correlation between the cognitive performance and AChE activity of the ENA713 treated mice (not shown).

4. Discussion

The results of this study show that the memory impairments associated with apoE deficiency can be ameliorated by two cholinomimetic treatments, the M1 agonist AF150(S) and the AChE inhibitor ENA713. The two treatments however, seem to differ in their mechanisms of action. Treatment with AF150(S) induced an increase in cortical and hippocampal AChE activity in the apoE-deficient mice while having almost no effect on that of control mice. On the other hand, treatment with ENA713 reduced AChE activity in control mice but that of the apoE-deficient mice was unaffected.

The behavioral experiments with the AChE inhibitor were performed each day about 20 h after treating the mice. Thus, since the effect of ENA713 in rodents lasts only 6 h with this dose [2, 16], it is unlikely that the observed decreases in the levels of cortical and hippocampal AChE activities are due to inhibition of AChE by residual levels of ENA713. This assertion is also supported by the finding that AChE levels in other brain areas such as the striatum are not affected. This then suggests that the histological, as well as the behavioral effects of ENA713 result from the prolonged daily treatments. The AF150(S) behavioral experiments were performed shortly after the daily administration of the drug.

However, in view of the pronounced effects of AF150(S) on both brain AChE activities and choline acetyltransferase (ChAT) levels of the apoE-deficient mice [5], it is likely that the effects of AF150(S) are also due to chronically activated mechanisms.

The biochemical mechanisms by which AF150(S) and ENA713, reverse the working memory deficits in the apoE-deficient mice, are not yet known. We have previously shown that AF150(S) elevates the brain ChAT and AChE levels of apoE-deficient mice back to those of the control mice [5]. One interpretation is that the effect of AF150(S) on working memory is due to restoration of brain cholinergic synaptic efficacy through activation of M1 muscarinic receptors. The effect of ENA713 on ChAT levels in apoE-deficient mice have not yet been determined. However, it has been shown in other models of cholinergic deficiency that ChAT levels are restored by prolonged treatment with ENA713 [12, 13]. It is thus likely that ENA713, like AF150(S) increases the efficacy of brain cholinergic synapses. However, as the effects of ENA713 and AF150(S) on brain AChE activity are different, we may conclude that the detailed cellular mechanisms by which these compounds reverse brain cholinergic deficiencies are not the same. This could be due in part to the finding that M1 muscarinic activation stimulates the expression of the AChE gene [14], and that apoE-deficient mice seem to have raised levels of M1 muscarinic receptors (in preparation).

In conclusion, this study revealed that cholinomimetic treatment of apoE-deficient mice either by an M1 muscarinic agonist or by an AChE inhibitor, ameliorates their memory deficits and suggests that

these effects are mediated by different cellular mechanisms.

References

- [1] Chapman S., Michaelson D.M., Specific neurochemical derangements of brain projecting neurons in apolipoprotein E-deficient mice, *J. Neurochem.* 70 (1998) 708–714.
- [2] Enz A., Amstutz R., Hoffman A., Gmelin G., Kelly P.H., Pharmacological properties of the preferentially centrally-acting acetylcholinesterase inhibitor SDZENA713, in: Kewitz H., Thompson T., Bickel U (Eds.), *Pharmacological interventions on central cholinergic mechanisms in senile dementia*, Zuckschwerdt, Munich, 1989, pp. 271–277.
- [3] Fisher A., Heldman E., Gurwitz D., Haring R., Meshulam H., Brandeis R., Sapir M., Marciano B., Barak D., Vogel Z., Karton Y., AF150(S) and AF151(S), new M₁ agonists, mediate M₁ selective signalling neurotrophic effects and restore AF64A cognitive deficits in rats, *Soc. Neurosci. Abstr.* 19 (1993) 1767.
- [4] Fisher A., Muscarinic agonists for the treatment of Alzheimer's disease: progress and perspectives, *Exp. Opin. Invest. Drugs.* 6 (1997) 1395–1411.
- [5] Fisher A., Brandeis R., Chapman S., Pittel Z., Michaelson D.M., M₁ muscarinic agonist treatment reverses cognitive and cholinergic impairments of apolipoprotein E-deficient mice, *J. Neurochem.* (1998), in press.
- [6] Gordon I., Grauer E., Genis I., Sehayek E., Michaelson D.M., Memory deficits and cholinergic impairments in apolipoprotein E-deficient mice, *Neurosci. Lett.* 199 (1995) 1–4.
- [7] Giacobini E., New trends in cholinergic therapy for Alzheimer's disease: nicotinic agonist or cholinesterase inhibitors, *Progr. Brain Res.* 109 (1996) 311–323.
- [8] Karnovsky M.J., Roots L.A., A direct cloning thiocholine method for cholinesterases, *J. Histochem. Cytochem.* 121 (1964) 219–222.
- [9] Plump A.S., Smith J.D., Hayek T., Aalto-Setälä K., Walsh A., Vermyt J.G., Rubin E.M., Breslaw J.L., Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created in homologous recombination in E5 cells, *Cell* 16 (1992) 343–353.
- [10] Poirier J., Delisle M.-C., Quirion R., Aubert I., Farlow M., Lahiri D., Hsui S., Bertrand P., Nalbantoglu J., Gilfir B.M., Gauthier S., Apolipoprotein E4 allele as a predictor of cholinergic deficits and treatment outcome in Alzheimer's disease, *Proc. Natl. Acad. Sci. USA* 92 (1995) 12260–12264.
- [11] Roses A.D., Apolipoprotein E effects on the rate of Alzheimer's disease expression in beta-amyloid burden is a secondary consequence dependent on apoE genotype and duration of disease, *J. Neuropathol. Exp. Neurol.* 53 (1994) 429–431.
- [12] Tanaka K., Ogawa N., Asanuma M., Kondo Y., Mori A., Chronic administration of acetylcholinesterase inhibitor in the senescent rat brain, *Neurobiol. Aging.* 15: (1994) 721–725.
- [13] Tanaka K., Ogawa N., Mizukawa K., Asanuma M., Kondo Y., Nishibayashi S., Mori A., Acetylcholinesterase inhibitor ENA-713 protects against ischemia-induced decrease in pre- and postsynaptic cholinergic indices in the gerbil brain following transient ischemia, *Neurochem. Res.* 19 (1994) 117–122.
- [14] Von der Kammer H., Albrecht C., Mayhaus M., Nitsch R.M., Identification of neurotransmitter-induced genes: novel targets for the molecular pharmacology of Alzheimer's disease, in: *Proceedings of the 5th International Geneva/Springfield Symposium on Advances in Alzheimer's Therapy*, 1998, 116 p.
- [15] Weinstock M., Razin M., Chorev M., Tashma Z., Pharmacological activity of novel acetylcholinesterase agents of potential use in the treatment of Alzheimer's disease, in: Fisher A., Hanin I., Lachman C., (Eds.), *Advances in Behavioral Biology*, Plenum Press, 1986, pp. 539–551.
- [16] Weinstock M., Razin M., Chorev M., Enz A., Pharmacological evaluation of phenyl-carbamates as CNS-selective acetylcholinesterase inhibitors, *J. Neural. Transm. Suppl.* 43 (1994) 219–225.

Neuromuscular responses to pyridostigmine bromide in organotypic spinal cord-muscle culture

Rosemarie Drake-Baumann^{a, b}, Fredrick J. Seil^{a, b, c}, Peter S. Spencer^{b, d*}

^aNeurology Research, Department of Veterans, Affairs Medical Center; ^bDepartment of Neurology and ^cCell and Developmental Biology, School of Medicine, and ^dCenter for Research on Occupational and Environmental Toxicology, Oregon Health Sciences University, Portland, Oregon 97201, USA

Abstract — Pyridostigmine bromide (PB) promotes and then silences cholinergic muscle activity, and disrupts the junctional regions of muscle fibers and associated nerve terminals, in organotypic mouse spinal cord-muscle cultures continuously treated with low concentrations of the drug for up to 14 days. Spontaneous muscle activity is restored within 1 week of drug removal. (©Elsevier, Paris)

Résumé — Effets neuromusculaires de la pyridostigmine sur des cultures organotypiques de moëlle épinière et de muscle. Dans des cultures organotypiques de muscle et de moëlle épinière de souris, traitées par de faibles doses de bromure de pyridostigmine (PB) jusqu'à 14 jours, on observe une stimulation puis un blocage de l'activité musculaire et une désorganisation des régions jonctionnelles des fibres musculaires et des terminaisons nerveuses associées. L'activité musculaire spontanée se rétablit une semaine après suppression de la drogue. (©Elsevier, Paris)

pyridostigmine bromide / neurotoxicity / muscle contractility / junctional myopathy / organotypic tissue culture / Gulf War Unexplained Illness

1. Introduction

Pyridostigmine bromide is a carbamate inhibitor of acetylcholinesterase (AChE), the enzyme responsible for the hydrolysis of neurotransmitter acetylcholine (ACh) at mammalian central and peripheral nerve terminals, including the neuromuscular junction. PB has been used in the civilian setting for the treatment of myasthenia gravis (a muscle disorder featured by serum ACh receptor antibodies) and, for military use, as an organophosphate nerve agent antidote enhancer. PB inhibits a percentage of peripheral cholinesterases and prevents access of soman to the inhibited (protected) enzyme, which subsequently decarbamylates to yield active enzyme [8, 10, 13]. During the 1991 Gulf War, US and UK troops were issued a blister pack containing 21 30-mg tablets of PB to be self-administered every 8 h for 1–7 days while under threat of nerve agent attack. A large majority of ground personnel received at least one dose and probably up to the full 21 tablets dispensed [23]. A study of 41 650 US soldiers, 34 000 of whom had taken PB for 6–7 days, revealed that pathophysiological changes were reported by about one half of this population [17]. Symptoms in this group were more frequent than would be expected from clinical experience with myasthenia gravis, in which larger therapeutic doses (30–60 mg every 4–6 h) of PB are used for prolonged periods. Moreover, only minor drug effects were reported in

a 2-week, double-blind, placebo-controlled cross-over study of seven 18- to 24-year-old healthy males subjected to heat stress while receiving PB 30 mg orally t.i.d. for 7 consecutive days [4]. Recent murine studies suggest that both PB and acute stress (forced swimming) initially increase ACh synthesis [16].

Toxic side effects are recognized in patients who receive PB for the treatment of myasthenia gravis; these are usually attributable to the reversible peripheral anti-cholinesterase actions of the pyridostigmine of PB, a quaternary moiety that fails readily to traverse the blood-brain regulatory interface except in animals subjected to acute stress [9]. The bromide moiety, which competes with chloride ions especially in states of dehydration, should readily cross the blood-brain barrier and is an established cause of neurological and neuropsychiatric disease (bromism), including fatigue and diminution of muscle power that probably have a central origin [22]. There is neurophysiological evidence of a progressive terminal axonal dysfunction in motor nerves of myasthenic patients chronically treated with PB [3], but the etiology is unknown. There is also evidence from experimental animal studies that repeated treatment with large doses of PB, as well as long-term anticholinesterase therapy, results in pathological changes of muscle and motor nerve terminals at motor end plates [7, 11, 14, 15, 21].

The action of PB at the neuromuscular junction can be studied under controlled conditions using spinal cord explants which, when co-cultured with

* Correspondence and reprints

striated muscle explants, develop into a structurally and functionally coupled organotypic array that closely resembles tissue organization *in vivo*. Organotypic cultures have proved useful in studies of neuromuscular function and in dissecting neuromuscular responses to a wide range of chemicals that have dose- and time-dependent neurotoxic potential [6, 28]. The present study employs mouse cord-muscle cultures to examine the neurotoxic actions of PB at the nerve-muscle interface after short-term and continuous exposure to drug concentrations of therapeutic relevance.

2. Materials and methods

Experiments used organotypic mouse cord-muscle cultures that were allowed to mature in roller tubes. Mature cultures were treated for up to 14 days with PB in low concentration (3.8×10^{-6} M) or high concentration (38×10^{-6} M), sodium bromide (3.8×10^{-6} M), or maintained in PB-free nutrient fluid (control). The lower PB concentration produced 30–60% inhibition of human and canine erythrocyte AChE [18, 29], a level of enzyme inhibition comparable to that produced in human volunteers given oral PB 30 mg/8 h for 1 week [4].

Electrophysiological examination of cultures was performed weekly. The recording medium consisted of (in mM): 137 NaCl, 2.7 KCl, 1 MgCl₂, 1 CaCl₂, 0.15 NaH₂PO₄, 1.34 Na₂HPO₄, 5.9 NaHCO₃, 5.5 glucose and 10 HEPES, pH 7.4, at room temperature. Drugs were dissolved and incorporated into the medium or applied directly over muscle by microinjection; final calculated concentrations are given. Intracellular microelectrodes were filled with 4 M potassium acetate, pH 4.0 (Aldrich, Milwaukee, WI, USA).

Data were evaluated by multivariate analysis of variance and logistic regression (Systat, SPSS, Chicago, IL and S-Plus, StatSci, Inc., Seattle, WA, USA).

3. Results

Acute application of PB (3 µg/mL) to the bath solution rapidly (seconds) and reversibly enhanced the number and frequency of spontaneous muscle contractions. Continuous treatment with PB decreased muscle fiber activity as a function of calculated drug concentration and time (*figure 1*). Differences in recorded spontaneous muscle contractions of cultures treated with high calculated concentrations (10 µg/mL) versus low concentrations (1 µg/mL) were evident as early as 24 h of drug treatment. After 1 week of treatment, during which spontaneous muscle contractions progressively decreased, muscle fibers became less responsive to exogenous application of ACh (10^{-4} M). By 2 weeks, tested muscle fibers were largely silent (*figure 2*). However, direct stimulation through the recording electrode elicited muscle fiber contraction. Control cultures displayed spontaneous muscle contractions throughout the test

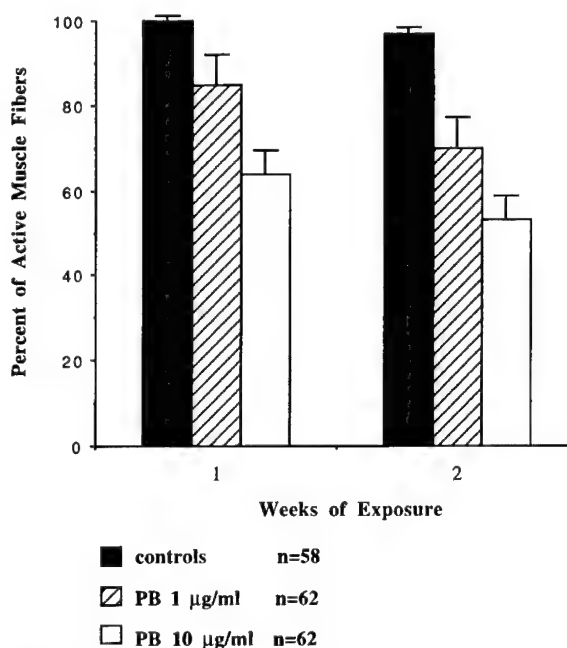


Figure 1. Relationship between percent of active muscle fibers, the dosage of pyridostigmine bromide (PB) and the drug treatment period.

period (*figure 2*). Removal of PB after a 2-week exposure resulted in a resumption of muscle activity. Within 1 week following withdrawal of PB, muscle fibers became functional, indicating that the neuromuscular effects of PB were reversible.

4. Discussion

Treatment with PB (1–10 µg/mL) induced the following sequential changes: i) an acutely reversible increase in muscle fiber activity consistent with drug-induced AChE inhibition; higher PB concentrations (200 mM–1 mM) are also reported to exert weak agonist effects on ACh receptors [1, 24]; ii) a reduction of sensitivity to exogenous acetylcholine consistent with an early desensitization of ACh receptors; iii) a progressive reduction of spontaneous neuromuscular activity without loss of muscle function; and iv) eventual recovery of spontaneous muscle activity following drug withdrawal. Since comparable pathophysiological changes fail to develop in cultures treated with sodium bromide, these neurotoxic phenomena are most likely direct anticholinesterase actions of the pyridostigmine moiety of PB at the neuromuscular junction. Recent studies of central cholinceptors suggest that PB-induced blockade of AChE produces an initial increase in ACh followed by a long-lasting decrease, an effect mediated by trans-

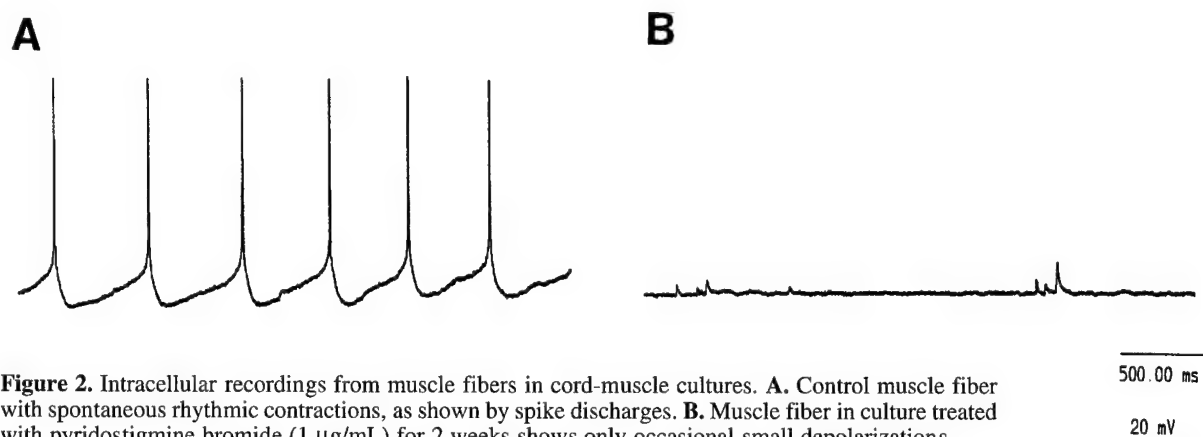


Figure 2. Intracellular recordings from muscle fibers in cord-muscle cultures. **A.** Control muscle fiber with spontaneous rhythmic contractions, as shown by spike discharges. **B.** Muscle fiber in culture treated with pyridostigmine bromide (1 µg/mL) for 2 weeks shows only occasional small depolarizations.

mitter-stimulated induction of mRNA levels encoding the early immediate transcription factor *c-fos* [16].

Organotypic cord-muscle cultures faithfully reproduce the timing, pattern, and spatial-temporal sequence of functional and structural changes induced by a variety of chemicals which display neurotoxic potential in animals and humans [28]. For example, continuous treatment of cord-muscle cultures with 2,5-hexanedione (2,5-HD), the agent responsible for induction of sensori-motor peripheral neuropathy in humans and animals repeatedly exposed to *n*-hexane, causes retrograde giant axonal degeneration of motor axons with consequent muscle denervation and silencing of spontaneous muscle fiber activity in a time course that matches similar changes in 2,5-HD-exposed animals [30]. In similar vein, characteristic changes would be expected at the human neuromuscular junction upon continuous exposure to a concentration of PB comparable to that achieved in cultures treated for 14 days with 1 µg/mL PB.

Neuropathological changes localized to neuromuscular junctions have been observed with a number of carbamate (PB, physostigmine) and organophosphate (tabun, sarin, soman) AChE inhibitors; this seems primarily to involve the muscle fiber and only secondarily affects the motor nerve terminal [7, 12]. This 'junctional myopathy' appears to be initiated by sustained accumulation of ACh at the motor endplate; this causes an increase in sarcoplasmic reticulum $[Ca^{2+}]$ that triggers events leading to muscle fiber necrosis [5, 19, 20, 22, 25, 27]. Neuromuscular junctions in the diaphragm (used here) are among the most severely affected in animals treated with anticholinesterase agents, and pathological changes are also prominent in soleus, gastrocnemius, and quadriceps muscles. Morphological alterations include presynaptic mitochondrial swelling, withdrawal of nerve terminals from the

postjunctional muscle membrane, and invasion of the synaptic cleft by Schwann cell processes. These characteristic ultrastructural changes may appear within hours of high-dose anticholinesterase drug administration, progress over a few days, and resolve within a couple of weeks [5]; they have also been observed after 7–14 days of continuous PB infusion, even at dosage rates as low as 9 µg/h [15]. Development of junctional myopathy is blocked by curare [31] and seems to depend on the degree and duration of AChase inhibition.

Junctional myopathy is likely to underly a life-threatening but spontaneously reversible (so-called intermediate) syndrome of proximal limb and intercostal muscle weakness that follows within days or longer of the successful treatment and resolution of a severe chemically-induced cholinergic crisis [26]. Since delayed-onset proximal muscle weakness was not reported by Gulf War veterans who experienced acute cholinergic effects from use of PB as a nerve-gas-antidote enhancer [17], it seems unlikely that cholinergic neuroexcitation in this setting was sufficient to induce a clinically significant junctional myopathy. Additionally, while muscle fatigue would predictably be associated with PB-induced neuromuscular damage, and fatigue is a prominent component of the symptom cluster reported by Gulf War veterans with unexplained illness, neurological examination has failed to reveal evidence of proximal muscle weakness typical of myopathy [2]. The reversibility of the PB-induced neuromuscular lesion in vitro as in vivo would also suggest that symptoms of fatigue which persist in Gulf War veterans years after exposure to PB are causally unrelated to the phenomenon observed in cord-muscle cultures continuously treated with PB. Involvement of motor nerve terminals at PB-affected neuromuscular junctions might explain neurophysiological evidence

of temporally progressive terminal axonal dysfunction in motor nerves of myasthenic patients chronically treated with PB [3].

Acknowledgments

Supported by a grant from the U.S. Department of Veterans Affairs to the Portland Environmental Hazards Research Center, a joint initiative of the Portland Veterans' Affairs Medical Center and the Center for Research on Occupational and Environmental Toxicology, Oregon Health Sciences University.

References

- [1] Akaike A., Ikeda S.R., Brookes N., Pascuzzo G.J., Rickett D.L., Albuquerque E.X., The nature of the interactions of pyridostigmine with the nicotinic acetylcholine receptor-ionic channel complex. II. Patch clamp studies, *Mol. Pharmacol.* 25 (1984) 102–112.
- [2] Amato A.A., McVey A., Cha C., Matthews E.C., Jackson C.E., Kleingunther R., Worley L., Cornman E., Kagan-Hallet K., Evaluation of neuromuscular symptoms in veterans of the Persian Gulf War, *Neurology* 48 (1997) 4–12.
- [3] Ballantyne J.P., Hansen S., Computer method for the analysis of evoked motor unit potentials. I. Control subjects and patients with myasthenia gravis, *J. Neurol. Neurosurg. Psychiatry* 37 (1974) 1187–1194.
- [4] Cook J.E., Kolka M.A., Wenger B.C., Chronic pyridostigmine bromide administration: side effects among soldiers working in a desert environment, *Milit. Med.* 157 (1992) 250–254.
- [5] Dettbarn W.D., Pesticide induced muscle necrosis: mechanisms and prevention, *Fund. Appl. Toxicol.* 4 (1984) S18–S26.
- [6] Ecob M.S., The application of organotypic nerve cultures to problems in neurology with special reference to their potential use in research into neuromuscular diseases, *J. Neurol. Sci.* 58 (1983) 1–15.
- [7] Engel A.G., Lambert A.H., Santa T., Study of long-term anticholinesterase therapy. Effects on neuromuscular transmission and on motor end-plate fine structure, *Neurology* 23 (1973) 1273–1281.
- [8] French M.C., Wetherell J.R., White P.D.T., The reversal by pyridostigmine of neuromuscular block produced by soman, *J. Pharm. Pharmacol.* 31 (1979) 290–294.
- [9] Friedman A., Kaufer D., Shemer J., Hendler I., Soreq H., Turkaspa I., Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response, *Nature Med.* 2 (1997) 1382–1385.
- [10] Gall D., The use of therapeutic mixtures in the treatment of cholinesterase inhibition, *Fund. Appl. Pharmacol.* 1 (1981) 214–216.
- [11] Gebbers J.O., Lotscher M., Kobel W., Portmann R., Laissue J.A., Acute toxicity of pyridostigmine in rats: histological findings, *Arch. Toxicol.* 58 (1986) 271–275.
- [12] Glazer E.J., Baker T., Riker W.F., Jr., The neuropathology of DFP at cat soleus neuromuscular junction, *J. Neurocytol.* 7 (1978) 741–758.
- [13] Gordon J.J., Leadbeater L., Maidment M.P., The protection of animals against organophosphate poisoning by pretreatment with a carbamate, *Toxicol. Appl. Pharmacol.* 43 (1978) 207–216.
- [14] Hudson C.S., Foster R.E., Kahng M.W., Neuromuscular toxicity of pyridostigmine bromide in the diaphragm, extensor digitorum longus, and soleus muscles of the rat, *Fund. Appl. Toxicol.* 5 (1985) S260–S269.
- [15] Hudson C.S., Foster R.E., Kahng M.W., Ultrastructural effects of pyridostigmine on neuromuscular junctions in rat diaphragm, *Neurotoxicology* 7 (1986) 167–186.
- [16] Kaufer D., Friedman A., Seidman S., Soreq H., Acute stress facilitates long-lasting changes in cholinergic gene expression, *Nature* 393 (1998) 373–377.
- [17] Keeler J.R., Hurst C.G., Dunn M.A., Pyridostigmine used as a nerve agent pretreatment under wartime conditions, *J. Am. Med. Assoc.* 266 (1991) 693–695.
- [18] Kluwe W.W., Page J.G., Toft J.D., Ridder W.E., Chung H., Pharmacological and toxicological evaluation of orally administered pyridostigmine in dogs, *Fund. Appl. Toxicol.* 14 (1990) 40–53.
- [19] Leonard J.P., Salpeter M.M., Agonist-induced myopathy at the neuromuscular junction is mediated by calcium, *J. Cell Biol.* 82 (1979) 811–819.
- [20] Meshul C.K., Calcium channel blocker reverses anticholinesterase-induced myopathy, *Brain Res.* 497 (1989) 142–148.
- [21] Meshul C.K., Boyne A.F., Deshpande S.S., Albuquerque E.X., Comparison of the ultrastructural myopathy induced by anticholinesterase agents at the end plates of rat soleus and extensor muscles, *Exp. Neurol.* 89 (1985) 96–114.
- [22] Moses H., Illrd, Klawans, H.L., Bromide intoxication, in: Vinken P.J., Bruyn G.W. (Eds.), *Intoxications of the Nervous System*, North-Holland, Amsterdam, 1979, pp. 291–318.
- [23] National Institutes of Health Technology Assessment Workshop Panel, The Persian Gulf experience and health, *J. Am. Med. Assoc.* 272 (1994) 391–396.
- [24] Pascuzzo G.J., Akaike A., Maleque M.A., Shaw K.P., Arons-tam R.S., Rickett D.L., Albuquerque E.X., The nature of the interactions of pyridostigmine with the nicotinic acetylcholine receptor-ionic complex. I. Agonist, desensitizing, and binding properties, *Mol. Pharmacol.* 25 (1984) 92–101.
- [25] Patterson G.T., Gupta R.C., Misulis K.E., Dettbarn W.D., Prevention of diisopropylphosphorofluoridate (DFP)-induced skeletal muscle fiber lesions in the rat, *Toxicology* 48 (1988) 237–244.
- [26] Senanayake N., Karaliedde I., Neurotoxic effects of organophosphorus insecticides: an intermediate syndrome, *New Engl. J. Med.* 316 (1987) 761–763.
- [27] Sket D., Dettbarn W.D., Clinton M.E., Misulis K.E., Sketelj J., Cucek D., Brzin M., Prevention of diisopropylphosphorofluoridate-induced myopathy by botulinum toxin type A blockage of quantal release of acetylcholine, *Acta Neuropathol. (Berl.)* 82 (1991) 134–142.
- [28] Spencer P.S., Crain S.M., Bornstein M.B., Peterson E.R., Van de Water T., Chemical neurotoxicity: Detection and analysis in organotypic cultures of sensory and motor systems, *Food Chem. Toxicol.* 24 (1986) 539–544.
- [29] Thomsen T., Zendeh B., Fischer J.P., Kewitz H., In vitro effects of various cholinesterase inhibitors on acetyl- and butyrylcholinesterase of healthy volunteers, *Biochem. Pharmacol.* 41 (1991) 139–141.
- [30] Veronesi B., Peterson E.R., Bornstein M.B., Spencer P.S., Ultrastructural studies of the dying-back process. VI. Examination of nerve fibers undergoing giant axonal degeneration in organotypic culture, *J. Neuropathol. Exp. Neurol.* 42 (1983) 153–165.
- [31] Wecker L., Dettbarn W.D., Paraoxon-induced myopathy: Muscle specificity and acetylcholine involvement, *Exp. Neurol.* 51 (1976) 281–291.

Contribution of nicotinic receptors to the function of synapses in the central nervous system: The action of choline as a selective agonist of $\alpha 7$ receptors

Edson X. Albuquerque^{a, b}, Edna F.R. Pereira^a, Maria F.M. Braga^{a, b},
Manickavasagam Alkondon^a

^aDepartment of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, MD 21201, USA

^bDepartment of Basic and Clinical Pharmacology, Institute of Biomedical Sciences, Center of Health Sciences, Federal University of Rio de Janeiro, Rio de Janeiro, RJ 21941, Brazil

Abstract — The $\alpha 7$ -nicotinic receptor (nAChR)-selective agonist choline and nAChR-subtype-selective antagonists led to the discovery that activation of both $\alpha 7$ and $\alpha 4\beta 2$ nAChRs located in CA1 interneurons in slices taken from the rat hippocampus facilitates the tetrodotoxin (TTX)-sensitive release of γ -aminobutyric acid (GABA). Experiments carried out in cultured hippocampal neurons not only confirmed that preterminal $\alpha 7$ and $\alpha 4\beta 2$ nAChRs modulate the TTX-sensitive release of GABA, but also demonstrated that evoked release of GABA is reduced by rapid exposure of the neurons to acetylcholine (ACh, 10 μ M–1 mM) in the presence of the muscarinic receptor antagonist atropine (1 μ M). This effect of ACh, which is fully reversible and concentration-dependent, is partially blocked by superfusion of the cultured neurons with external solution containing either the $\alpha 7$ -nAChR-selective antagonist methyllycaconitine (MLA, 1 nM) or the $\alpha 4\beta 2$ -nAChR-selective antagonist dihydro- β -erythroidine (DH β E, 100 nM). A complete blockade of ACh-induced reduction of evoked release of GABA was achieved only when the neurons were perfused with external solution containing both MLA and DH β E, suggesting that activation of both $\alpha 7$ and $\alpha 4\beta 2$ nAChRs modulates the evoked release of GABA from hippocampal neurons. Such mechanisms may account for the apparent involvement of nAChRs in the psychological effects of tobacco smoking, in brain disorders (e.g., schizophrenia and epilepsy), and in physiological processes, including cognition and nociception. (©Elsevier, Paris)

Résumé — Contribution des récepteurs nicotiniques à la fonction des synapses dans le système nerveux central.: action de la choline comme agoniste des récepteurs $\alpha 7$. En utilisant la choline comme agoniste sélectif du récepteur nicotinique (nAChR) de type $\alpha 7$ et des antagonistes sélectifs des différents sous-types de nAChR, nous avons découvert que l'activation de récepteurs $\alpha 7$ et $\alpha 4\beta 2$ localisés sur des interneurons de CA1 dans des tranches d'hippocampe de rat facilite la libération d'acide γ -aminobutyrique (GABA) sensible à la tétrodotoxine (TTX). Des expériences effectuées sur des neurones d'hippocampe en culture ont confirmé que des récepteurs $\alpha 7$ et $\alpha 4\beta 2$ localisés sur des interneurons de CA1 dans des tranches d'hippocampe de rat facilite la libération d'acide γ -aminobutyrique (GABA) sensible à la tétrodotoxine (TTX). modulent la libération de GABA sensible à la TTX, et ont aussi démontré que la libération évoquée du GABA est réduite par une exposition rapide des neurones à l'acétylcholine (ACh, 10 μ M–1 mM), en présence d'atropine, un antagoniste muscarinique (1 μ M). Cet effet de l'ACh, qui est entièrement réversible et dépend de la concentration, est bloqué par superfusion des neurones avec une solution de méthyllycaconitine (MLA, 1 nM), un antagoniste sélectif d' $\alpha 7$, ou de dihydro- β -érythroidine (DH β E, 100 nM), un antagoniste sélectif d' $\alpha 4\beta 2$. On n'obtient la suppression complète de la réduction de libération de GABA induite par l'ACh que lorsque les neurones sont perfusés à la fois avec MLA et DH β E, ce qui suggère que l'activation des deux types de récepteurs, $\alpha 4\beta 2$, module la libération des GABA par les neurones d'hippocampe. De tels mécanismes peuvent expliquer le fait que des récepteurs nicotiniques paraissent impliqués dans les effets psychologiques de l'action de fumer du tabac, dans des désordres cérébraux (par exemple le schizophrénie et l'épilepsie) et dans des processus physiologiques comme la cognition et la nociception. (©Elsevier, Paris)

choline / nicotinic receptors / slices / hippocampus / GABA

Abbreviations: α -BGT, α -bungarotoxin; ACh, acetylcholine; ACSF, artificial cerebrospinal fluid; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-7-nitroquinoline-2,3-dione; CNS, central nervous system; DH β E, dihydro- β -erythroidine; EGTA, ethyleneglycol bis(β -amino-ethyl ether)-N,N-tetraacetic acid; GABA, γ -aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPSCs, inhibitory postsynaptic currents; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; OPs, organophosphates; PSCs, postsynaptic currents; TTX, tetrodotoxin.

1. Introduction

It is generally acknowledged that the hippocampus, an area of the central nervous system (CNS) that is involved in processing cognitive functions [20], is very sensitive to cholinergic modulation, and that the density of neuronal nicotinic acetylcholine receptors (nAChRs) in the hippocampus is severely diminished in diseases characterized by learning and memory impairment (e.g., Alzheimer's disease) [21].

Thus, the developing hippocampus has become the focus of our research interest with regard to characterization of the nAChR function. One of the major hindrances to addressing nAChR function in the brain was the lack of specific agonists and antagonists for each of the receptor subtypes. This problem was further aggravated by the fast kinetics of inactivation of some of the neuronal nicotinic receptors, particularly of those composed of the $\alpha 7$ subunit. The field has now advanced immensely, and many of these problems have been overcome (reviewed in [2]).

In many areas of the brain, nAChRs have been shown to facilitate the release of neurotransmitters [4, 12, 13, 17, 18] (see also review [24] and references cited therein). In general terms, modulation of transmitter release by presynaptic nAChRs (i.e., nAChRs present in synaptic terminals) is insensitive to blockade by the Na⁺-channel blocker tetrodotoxin (TTX), whereas modulation of transmitter release by preterminal nAChRs (i.e., nAChRs present in axons) depends on propagation of action potentials and is, therefore, sensitive to TTX. Identifying the neuronal nAChR subtype involved in modulating the release of a given neurotransmitter has been a more difficult task.

The introduction of a set of pharmacological and kinetic 'fingerprints' has been very useful in identifying the nAChR subtype that subserves a given nicotinic response [3, 7]. It has been suggested that fast-desensitizing nicotinic responses sensitive to blockade by α -bungarotoxin (α -BGT) or methyllycaconitine (MLA) are mediated by $\alpha 7$ -containing nAChRs, and that slowly desensitizing nicotinic responses sensitive to blockade by dihydro- β -erythroidine (DH β E) and mecamylamine are mediated by $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChRs, respectively [3, 7]. The recent discovery that choline modulates the function and expression of $\alpha 7$ nAChRs also introduced a key pharmacological tool to distinguish these from other nAChR subtypes [5, 19]. In cultured hippocampal neurons, choline fully activates $\alpha 7$ nAChR-mediated currents with an apparent EC₅₀ of 1.6 mM, does not evoke $\alpha 4\beta 2$ nAChR-mediated currents, and induces $\alpha 3\beta 4$ nAChR-mediated currents with 20% of the apparent efficacy of acetylcholine (ACh). In addition, when continuously applied to these neurons, choline, like other nicotinic agonists, desensitizes the $\alpha 7$ nAChRs subserving type IA currents with an IC₅₀ of 37 μ M [5]. Therefore, choline as a nicotinic agonist has the unique capability of providing substantial clues regarding the nAChR subtype subserving a nicotinic response.

Initial studies from our laboratory provided evidence that activation of nAChRs in CA1 interneurons results in a variety of responses, including

facilitation of γ -amino butyric acid (GABA) release [4]. In this paper, evidence is provided that the TTX-sensitive release of GABA from CA1 interneurons and from cultured hippocampal neurons is facilitated by activation of both $\alpha 7$ and $\alpha 4\beta 2$ nAChRs, and that field stimulation-induced release of GABA from hippocampal neurons in culture is inhibited by the activation of both nAChR subtypes. Choline, by acting as a selective $\alpha 7$ -nAChR-agonist, may have a role in controlling synaptic function in the mammalian CNS.

2. Materials and methods

2.1. Hippocampal slices

Slices of 250–300- μ m thickness were obtained from the hippocampus of 12–24-day-old Sprague-Dawley rats according to the procedure described earlier [4]. Slices were stored at room temperature in artificial cerebrospinal fluid (ACSF), which was bubbled with 95% O₂ and 5% CO₂ and had the following composition (in mM): NaCl 125; NaHCO₃ 25; KCl 2.5; NaH₂PO₄ 1.25; CaCl₂ 2; MgCl₂ 1; and glucose 25.

2.2. Cell culture

Primary cultures of hippocampal neurons were done as described previously [3].

2.3. Electrophysiological recordings

Whole-cell currents were recorded from the soma of CA1 interneurons in slices and from the soma of cultured hippocampal neurons according to the standard patch-clamp technique [15], using an LM-EPC7 patch-clamp system (List Electronic, Darmstadt, Germany). The signals were filtered at 2 kHz and either recorded on a video tape recorder for later analysis or directly sampled by a microcomputer using the pCLAMP6 program (Axon Instruments, Foster City, CA, USA). Neurons in slices were superfused with ACSF at 2 mL/min, and cultured neurons were superfused with external solution, which had the following composition (in mM): NaCl, 165; KCl, 5; CaCl₂ 2; glucose 10; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5 (pH adjusted to 7.3 with NaOH; 340 mOsm). Atropine (1 μ M) was added to the ACSF and to the external solution to block the muscarinic receptors. Patch pipettes were pulled from borosilicate glass capillary (1.2-mm outer diameter), and when filled with the internal solution had resistances between 2 and 6 M Ω . The internal solution consisted of (in mM): ethyleneglycol bis (β -amino-ethyl ether)-N,N-tetraacetic acid (EGTA) 10; HEPES 10; CsCl 80; CsF 80 (pH was adjusted to 7.3 with CsOH; 340 mOsm). All recordings were performed at room temperature. Data analysis was done using the pCLAMP6 program.

2.4. Field stimulation of hippocampal neurons in culture

A glass-mounted electropolished bipolar platinum microelectrode system (specially designed and assembled in our laboratory) was used to stimulate neurons synaptically connected to the neuron under study in culture. Evoked GABA-mediated inhibitory postsynaptic currents (IPSCs) were recorded in the presence of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 6-cyano-7-nitroquinoline-2,3-dione (CNQX; 10 μ M). To avoid the Na^+ current transients that interfere with the synaptic currents, all experiments were carried out at positive membrane potentials. A supramaximal (~11 volt), 20- μ s pulse was applied at a rate of 1 Hz via the bipolar electrode. Stable responses could be obtained during 30 to 60 min of recording.

2.5. Drugs used

Acetylcholine chloride, choline chloride, dimethylsulfoxide (DMSO), tetrodotoxin, γ -aminobutyric acid, picrotoxin, and atropine sulfate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). α -Bungarotoxin was purchased from Biotoxins Inc. Methyllycaconitine citrate was a gift from Professor M.H. Benn (Department of Chemistry, University of Calgary, Alberta, Canada). Dihydro- β -erythroidine.HBr was a gift from Merck, Sharp & Dohme (Rahway, NJ, USA). A 250 mM stock solution of picrotoxin was made in DMSO. Stock solutions of all other drugs were made in distilled water. Further dilutions were made in either ACSF (for experiments in slices) or external solution (for experiments in cultured neurons).

3. Results

3.1. TTX-sensitive GABA release from CA1 interneurons is facilitated by activation of $\alpha 7$ and $\alpha 4\beta 2$ nAChRs

Three components could be identified in nicotinic responses recorded from CA1 interneurons rapidly exposed to equipotent, nearly saturating concentrations of ACh and of the $\alpha 7$ -nAChR-selective agonist choline. Two of these components, referred to as postsynaptic currents (PSCs) and fast current transients, depended on the propagation of action potentials, and, were, therefore sensitive to blockade by the Na^+ -channel blocker TTX (*figure 1*). The third component had the slowest rising and decaying phases and consisted of a TTX-insensitive event, which is referred to as the nicotinic current. The PSCs were the consequence of agonist-induced facilitation of GABA release, because they could be blocked by the GABA_A-receptor antagonist picrotoxin (100 μ M). Also, evidence has been provided that nicotinic currents and fast current transients were triggered by activation of nAChRs present on the neuron under study, whereas PSCs were triggered by acti-

vation of nAChRs present in presynaptic neurons [4, 6].

Fast-decaying nicotinic currents accompanied by GABA-mediated PSCs and fast current transients were observed in 68% of the neurons exposed to choline (10 mM); only approximately 17% of such neurons showed similar responses to ACh (1 mM). These responses, being evoked by the $\alpha 7$ nAChR-selective agonist choline and blocked by the selective $\alpha 7$ nAChR antagonists MLA (50 nM) and α -BGT (100 nM) (*figure 1*), were likely to be subserved by $\alpha 7$ nAChRs. It should be pointed out that the concentration of MLA needed to block these responses was considerably higher than that used to block $\alpha 7$ nAChR-mediated responses recorded from fetal hippocampal neurons in culture. Such a difference in the sensitivity of $\alpha 7$ nAChRs to MLA has been reported in previous studies [9] and suggests that the $\alpha 7$ nAChRs may exist in the hippocampus in multiple heteromeric and/or homomeric isoforms that change along with development. The question of whether these isoforms would also show differential sensitivity to the $\alpha 7$ -nAChR-selective agonist choline remains open.

Nicotinic responses apparently resulting from summation of large numbers of PSCs, alone or accompanied by a small slowly decaying nicotinic current, were observed in response to ACh in about 77% of the interneurons, and were sensitive to blockade by the $\alpha 4\beta 2$ -nAChR-selective antagonist DH β E (*figure 1*), but not by MLA or α -BGT. These DH β E-sensitive responses were likely to be subserved by $\alpha 4\beta 2$ nAChRs. Our findings, therefore, not only confirm those of a recent study showing that functional $\alpha 7$ nAChRs are present in CA1 interneurons [10], but extend the results by demonstrating that $\alpha 4\beta 2$ nAChRs are also present in preterminal sites of these neurons, and that activation of both receptor subtypes can facilitate the TTX-sensitive release of GABA.

3.2. TTX-sensitive and field-stimulation-evoked release of GABA from cultured hippocampal neurons is modulated by activation of $\alpha 7$ and $\alpha 4\beta 2$ nAChRs

A 1-s pulse application of ACh (1–1000 μ M) to hippocampal neurons in culture increased the frequency of PSCs, which were not sensitive to blockade by the AMPA-receptor antagonist CNQX (10 μ M), but were blocked by the GABA_A-receptor antagonist picrotoxin (100 μ M). In agreement with the results obtained from hippocampal slices, the blockade of the ACh-induced, GABA-mediated PSCs by TTX (150 nM) indicated that these responses resulted from activation of nAChRs located in preterminal sites or on the somato-dendritic region of presynaptic GABAergic neurons.

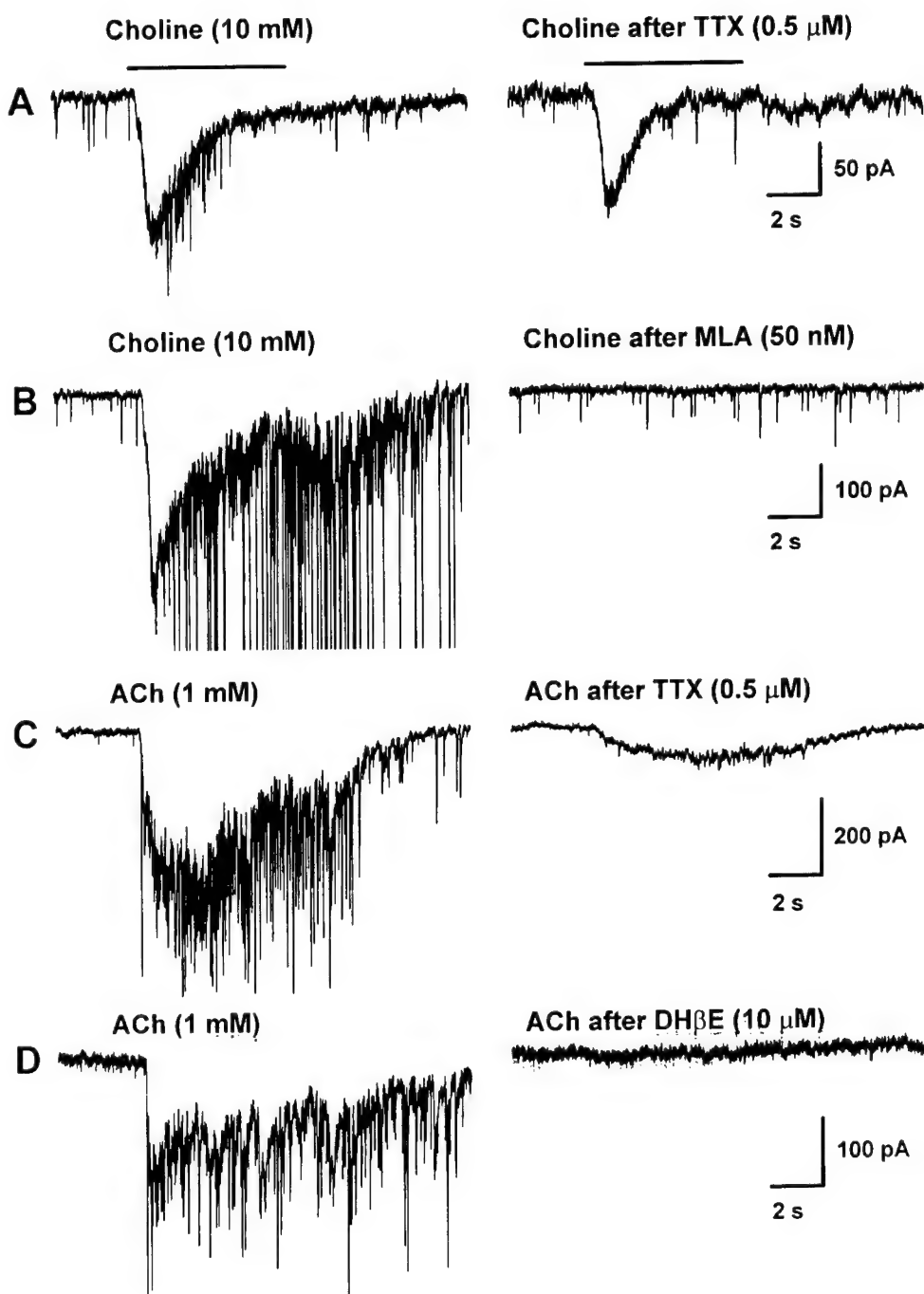


Figure 1. Nicotinic responses of CA1 interneurons. Responses recorded from four interneurons (labeled A–D) are shown. In **A**, choline induced a rapidly decaying whole-cell inward current accompanied by PSCs. Superfusion of the neurons with TTX ($0.5\ \mu\text{M}$) abolished choline-induced PSCs, but not the nicotinic current. In **B**, choline evoked nicotinic inward current accompanied by PSCs and fast current transients. The fast current transients are purposely clipped to unmask the other components. Superfusion with MLA ($50\ \text{nM}$) abolished all three components of the choline-evoked response. In **C**, ACh induced a response that consists mainly of PSCs. Superfusion of this neuron with TTX-containing solution blocked most of the response, leaving a small slowly decaying nicotinic current, which was due to the activation of nAChRs on the neuron from which the recordings were obtained. In **D**, ACh induced a similar response as in **C**, and this response was completely blocked by DH β E ($10\ \mu\text{M}$). The duration of the agonist pulse is indicated by the solid line above the top traces. The interneurons studied were located at $100\ \mu\text{m}$ (**A**), $150\ \mu\text{m}$ (**B** and **C**) and $125\ \mu\text{m}$ (**D**) from the midline of the pyramidal cell layer. Age of rats = 24 days (**A**, **C**, **D**) and 19 days (**B**). In all experiments the membrane potential was held at $-60\ \text{mV}$.

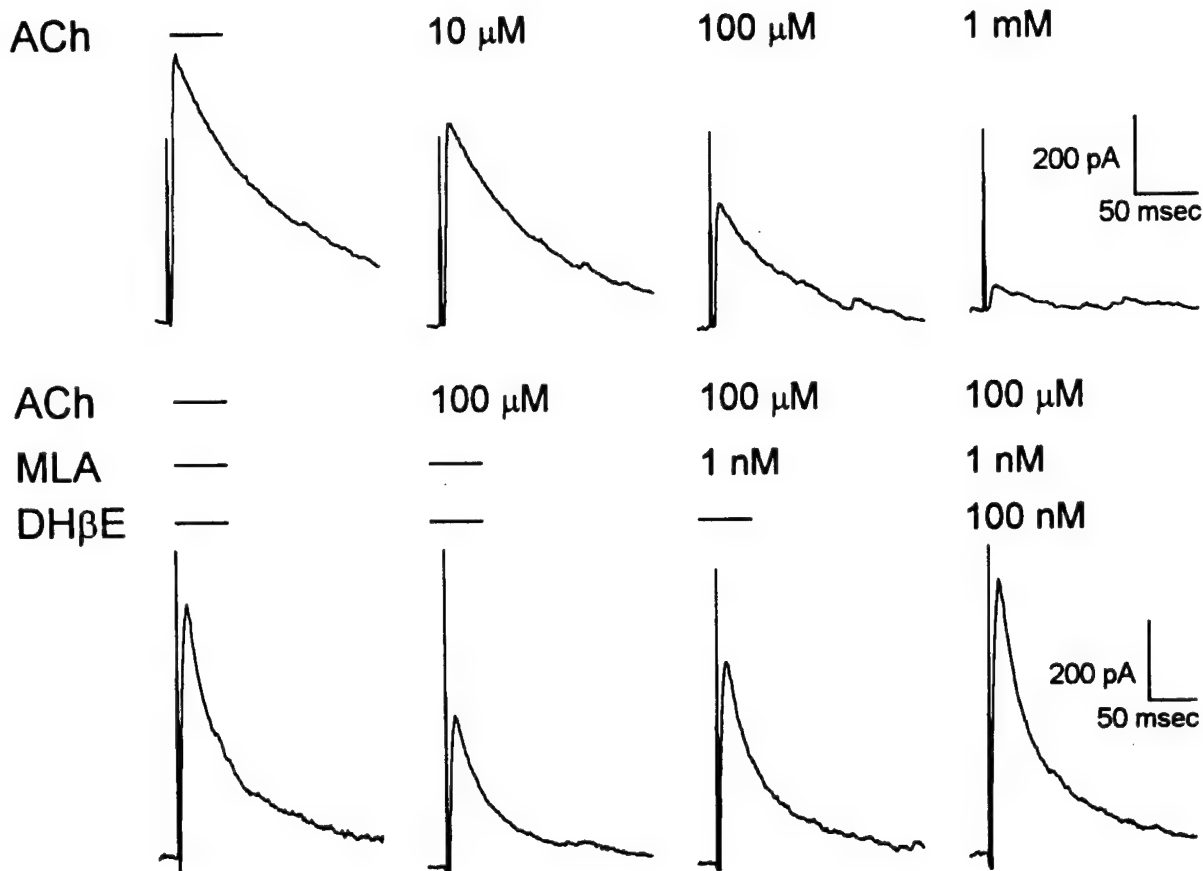


Figure 2. Acetylcholine reduces the amplitude of evoked IPSCs via activation of $\alpha 7$ and $\alpha 4\beta 2$ nAChRs. In 1-s intervals, neurons synaptically connected to the neuron under study in culture were stimulated with a supramaximal (~ 11 volt), 20- μ s pulse applied via the bipolar electroplated platinum electrode. After at least 10 control IPSCs were recorded from a single neuron, 1-s pulses of ACh (10 μ M–1 mM) were delivered to the neurons simultaneously with the electrical stimulus sent to the bipolar electrode. Notice that ACh reduces the amplitude of the evoked IPSCs. This effect of ACh, which was concentration dependent, was completely reversible. In the presence of MLA (1 nM), the ACh-induced reduction of the IPSC amplitude was partially blocked, whereas in the presence of both MLA and DH β E (100 nM) a complete blockade of the ACh effect was achieved.

In the presence of the muscarinic receptor antagonist atropine (1 μ M), rapid application (1–5 s) of ACh (10 μ M–1 mM) to the cultured hippocampal neurons, in addition to increasing the frequency of GABA-mediated PSCs (data not shown), reduced the amplitude of GABA-mediated IPSCs evoked by field stimulation of a neuron synaptically connected to the neuron under study (figure 2). It is likely that depolarization resulting from ACh-induced activation of nAChRs in presynaptic neurons generates areas of blockade of conduction of action potentials, and, ultimately, leads to the reduction of the evoked release of GABA. The effect of ACh on the evoked release was only partially blocked when the neurons

were perfused with physiological solution containing either MLA (1 nM) or DH β E (100 nM). A full blockade of the ability of ACh to reduce the evoked release of GABA was achieved by perfusing the neurons with physiological solution containing an admixture of MLA (1 nM) and DH β E (100 nM) (figure 2).

4. Discussion and conclusions

The $\alpha 7$ nAChR-selective agonist and nAChR-subtype-selective antagonists provided strong evidence that interneurons in the CA1 field of hippocampal

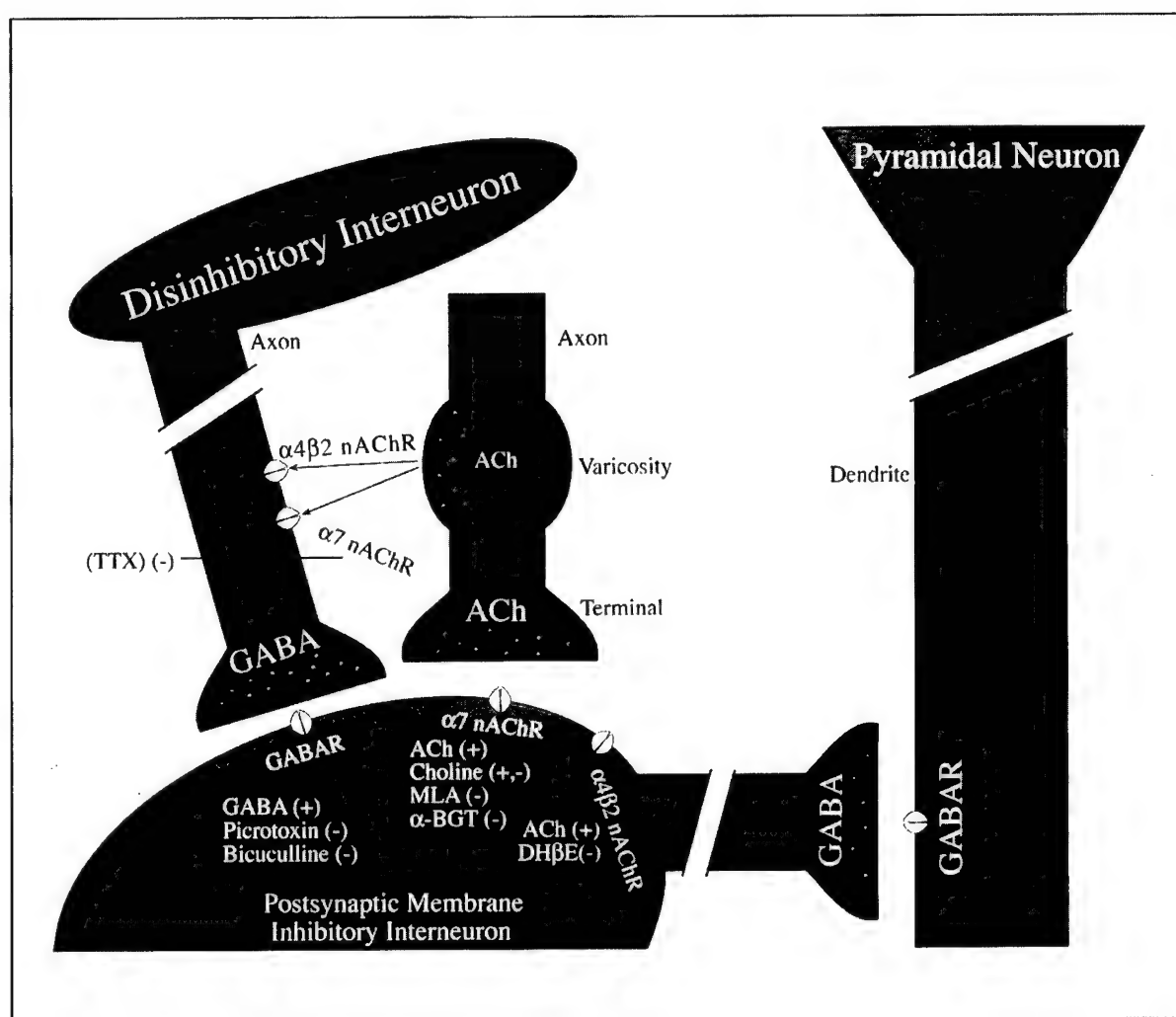


Figure 3. Scheme showing the possible sites of expression of functional $\alpha 7$ and $\alpha 4\beta 2$ nAChRs in CA1 interneurons. This hypothetical model is based on the results presented in this paper and in Alkonon et al. [6]. The cholinergic axon is likely to come from the septum, and GABAergic axons are likely to originate from other interneurons in different layers of the CA1 region. As depicted in the far left side of the scheme, activation of $\alpha 7$ and $\alpha 4\beta 2$ nAChRs located in presynaptic neurons can facilitate the TTX-sensitive release of GABA. Activation by GABA of GABA_A receptors present in the interneuron being studied would result in elicitation of GABA-mediated PSCs, which are sensitive to blockade by GABA_A-receptor antagonists. The nicotinic currents recorded from the interneuron under study would have been the consequence of the activation of nAChRs present in that neuron. Currents resulting from activation of $\alpha 7$ nAChRs would decay within the agonist pulse and would be equally sensitive to choline and ACh as agonists and to MLA and α -BGT as antagonists. On the other hand, currents resulting from activation of $\alpha 4\beta 2$ nAChRs would last longer than the agonist pulse, would be sensitive to ACh, but not to choline as an agonist, and would be blocked selectively by DH β E. By inference, as depicted in the far right side of the scheme, it is likely that modulation by nAChRs of the ongoing activity in the interneurons ultimately controls the excitability of the pyramidal neurons in the CA1 region. In this model, the sign (-) indicates that a given compound is capable of blocking the receptor activity, and the sign (+) indicates that the compound can activate a given receptor.

slices and cultured hippocampal neurons can express functional preterminal $\alpha 7$ and $\alpha 4\beta 2$ nAChRs and that activation of both nAChR subtypes facilitates the TTX-sensitive and blocks the evoked release of GABA (see figure 3) [6]. It is, thus, possible that

during cholinergic activation, ACh shuts off the GABAergic neuronal activity driven by presynaptic (glutamatergic) neurons and by itself modulates the release of GABA. This may be a unique mechanism by which cholinergic neurons take full control of ti-

ming of inhibitory inputs to hippocampal pyramidal neurons. It is noteworthy that the net result of activation of nAChRs appears to depend on the location of the receptor on the neuronal surface, because there is evidence that activation of $\alpha 7$ nAChRs present in glutamatergic terminals of hippocampal neurons facilitates the release of glutamate evoked by field stimulation of presynaptic neurons (Radcliffe and Dani, personal communication).

The neuronal circuitry in the hippocampus is highly complex, and interneurons are capable of innervating pyramidal neurons and neighboring interneurons [1, 14]. Thus, cholinergic nicotinic activation can produce both a direct inhibition and an indirect disinhibition of a pyramidal neuron, and timing of the two events could control the firing rate of a pyramidal neuron. This may represent a cholinergic mechanism that adjusts the activity of CA1 pyramidal neurons, and, consequently, the neuronal output from the hippocampus to higher brain centers. Disinhibition of CA3 pyramidal neuron activity by stimulation of septal GABAergic afferents has been recently demonstrated, and the importance of disinhibitory mechanisms in the hippocampal function has been stressed [23]. In the CA1 region, local GABAergic interneurons that are likely to be driven by various glutamatergic afferents have also been shown to have a disinhibitory function [1, 14]. Our results indicate that both $\alpha 7$ or $\alpha 4\beta 2$ nAChRs may be involved in controlling the overall activity of these disinhibitory cells, and provide direct support to the concept that a GABAergic mechanism could explain the relationship between $\alpha 7$ nAChR gene locus and attentional deficits in schizophrenia [11], and that between cholinergic deafferentation and kindling epileptogenesis [16].

Our results also indicate that choline, by controlling the function of $\alpha 7$ nAChRs in the CNS may have a role in modulating the activity of the CA1 interneurons, and, ultimately, the excitability of the CA1 pyramidal neurons. This finding calls for a review of our understanding of the mechanisms underlying the neurotoxic effects of compounds that inhibit cholinesterase, including organophosphates (OPs) used as pesticides or nerve agents. It has long been accepted that accumulation of non-hydrolyzed ACh in the synaptic cleft could lead to hyperactivation of the cholinergic systems in OP-induced intoxication. With the discovery of the selective agonist action of choline on $\alpha 7$ nAChRs [5] and of the involvement of these in modulation of transmitter release [4, 12, 13, 17, 18], it could be postulated that the neurotoxic effects of cholinesterase inhibitors arise from an imbalance between ACh and choline concentrations at the synaptic cleft.

Acknowledgments

The superb technical assistance of Ms. Mabel Zelle, Ms. Barbara Marrow, and Mr. Benjamin Cumming is gratefully acknowledged. The authors are also indebted to Ms. Mabel Zelle for her comments in the manuscript. This study was supported by USPHS grant NS 25296.

References

- [1] Acsády L., Görös T.J., Freund T.F., Different populations of vasoactive intestinal polypeptide-immunoreactive interneurons are specialized to control pyramidal cells or interneurons in the hippocampus, *Neuroscience* 73 (1996) 317-334.
- [2] Albuquerque E.X., Alkondon M., Pereira E.F.R., Castro N.G., Schrattenholz A., Barbosa C.T.F., Bonfante-Cabarcas R., Aracava Y., Eisenberg H.M., Maelicke A., Properties of neuronal nicotinic acetylcholine receptors: Pharmacological characterization and modulation of synaptic function, *J. Pharmacol. Exp. Ther.* 280 (1997) 1117-1136.
- [3] Alkondon M., Albuquerque E.X., Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. I. Pharmacological and functional evidence for distinct structural subtypes, *J. Pharmacol. Exp. Ther.* 265 (1993) 1455-1473.
- [4] Alkondon M., Pereira E.F.R., Barbosa C.T.F., Albuquerque E.X., Neuronal nicotinic acetylcholine receptor activation modulates γ -aminobutyric acid release from CA1 neurons of rat hippocampal slices, *J. Pharmacol. Exp. Ther.* 283 (1997) 1396-1411.
- [5] Alkondon M., Pereira E.F.R., Cortes W.S., Maelicke A., Albuquerque E.X., Choline is a selective agonist of $\alpha 7$ nicotinic acetylcholine receptors in rat brain neurons, *Eur. J. Neurosci.* 9 (1997) 2734-2742.
- [6] Alkondon M., Pereira E.F.R., Albuquerque E.X., Choline and selective antagonists identify multiple subtypes of nicotinic acetylcholine receptors that modulate GABA release from CA1 interneurons in rat hippocampal slices, *Abstr. Soc. Neurosci.* (24) 1998.
- [7] Alkondon M., Pereira E.F.R., Albuquerque E.X., Distinct nicotinic receptors (nAChRs) modulate GABA release from CA1 interneurons in rat hippocampal slices, *Abstr. Soc. Neurosci.* (24) 1998.
- [8] Cobb S.R., Buhl E.H., Halasy K., Paulsen O., Somogyi P., Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons, *Nature* 378 (1995) 75-78.
- [9] Drasdo A., Caufield M., Bertrand D., Bertrand S., Wonnacott S., Methyllycaconitine: a novel nicotinic antagonist, *Mol. Cell. Neurosci.* 3 (1992) 237-243.
- [10] Frazier C.J., Rollins Y.D., Breese C.R., Leonard S., Freedman R., Dunwiddie T.V., Acetylcholine activates an α -bungarotoxin-sensitive nicotinic current in rat hippocampal interneurons, but not pyramidal cells, *J. Neurosci.* 18 (1998) 1187-1195.
- [11] Freedman R., Coon H., Myles-Worsley M., Orr-Urtreger A., Olincy A., Davis A., Polymeropoulos M., Holik J., Hopkins J., Hoff M., Rosenthal J., Waldo M.C., Reimherr F., Wender P., Yaw J., Young D.A., Breese C.R., Adams C., Patterson D., Adler L.E., Kruglyak L., Leonard S., Byerler W., Linkage of neurophysiological deficit in schizophrenia to a chromosome 15 locus, *Proc. Natl. Acad. Sci. USA* 94 (1997) 587-592.

- [12] Gray R., Rajan A.S., Radcliffe K.A., Yakehiro M., Dani J.A., Hippocampal synaptic transmission enhanced by low concentrations of nicotine, *Nature* 383 (1996) 713–716.
- [13] Guo, J.Z., Tredway, T.L., Chiappinelli, V.A., Glutamate and GABA release are enhanced by different subtypes of presynaptic nicotinic receptors in the lateral geniculate nucleus, *J. Neurosci.* 18 (1998) 1963–1969.
- [14] Gulyás A.I., Hájos N., Freund T.F., Interneurons containing calretinin are specialized to control other interneurons in the rat hippocampus, *J. Neurosci.* 16 (1996) 3397–3411.
- [15] Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J., Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches, *Pflügers Arch.* 391 (1981) 85–100.
- [16] Kokaia M., Ferencz I., Leanza G., Elmér E., Metsis M., Kokaia Z., Wiley R.G., Lindval O., Immunolesioning of basal forebrain cholinergic neurons facilitates hippocampal kindling and perturbs neurotrophin messenger RNA regulation, *Neuroscience* 70 (1998) 313–327.
- [17] Léna C., Changeux J.P., Role of Ca^{2+} ions in nicotinic facilitation of GABA release in mouse thalamus, *J. Neurosci.* 17 (1997) 576–585.
- [18] McGehee D.S., Heath M.J.S., Gelber S., Devay P., Role L.W., Nicotinic enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors, *Science* (Wash. DC) 269 (1995) 1692–1696.
- [20] Papke R.L., Bencheriff M., Lippiello P., An evaluation of neuronal nicotinic acetylcholine receptor activation by quaternary nitrogen compounds indicates that choline is selective for the $\alpha 7$ subtype, *Neurosci. Lett.* 213 (1996) 201–204.
- [20] Petit T.L., The neurobiology of learning and memory: Elucidation of the mechanism of cognitive function, *NeuroToxicology* 5 (1988) 413–428.
- [21] Schröder H., Giacobini E., Struble R.G., Zilles K., Maelicke A., Nicotinic cholinergic neurons of the frontal cortex are reduced in Alzheimer's disease, *Neurobiol. Aging* 12 (1991) 259–262.
- [22] Spruston, N., Jonas, P., Sakmann, B., Dendritic glutamate receptor channels in rat hippocampal CA3 and CA1 pyramidal neurons, *J. Physiol. (Lond.)* 482 (1995) 325–352.
- [23] Tóth K., Freund T.F., Miles R., Disinhibition of rat hippocampal pyramidal cells by GABAergic afferents from the septum, *J. Physiol. (Lond.)* 500 (1997) 463–474.
- [24] Wonnacott S., Presynaptic nicotinic receptors, *TINS* 20 (1997) 92–98.

Chronic neurobehavioral and central and autonomic nervous system effects of Tokyo subway sarin poisoning

Kazuhito Yokoyama^a, Shunichi Araki^a, Katsuyuki Murata^a, Mariko Nishikitani^a,
Tetsu Okumura^b, Shinichi Ishimatsu^b, Nobukatsu Takasu^b

^a*Department of Public Health and Occupational Medicine, Graduate School of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan*

^b*Emergency Department, St. Luke's International Hospital, 9-1 Akashi-cho, Chuo-ku, Tokyo 104-0044, Japan*

Abstract — To evaluate delayed (prolonged) neurobehavioral and neurophysiological effects of acute sarin poisoning, nine male and nine female patients of the Tokyo subway sarin poisoning in Japan were examined by neurobehavioral tests, posttraumatic stress disorder (PTSD) checklist, brain evoked potentials, computerized static posturography, and electrocardiographic R-R interval variability, 6–8 months after the poisoning. Their serum cholinesterase activities on the day of the poisoning (March 20, 1995) were 13–131 (mean 72.1) IU/L. The results suggested delayed effects on psychomotor performance, the higher and visual nervous system and the vestibulo-cerebellar system with psychiatric symptoms resulting from PTSD. (©Elsevier, Paris)

Résumé — Effets chroniques comportementaux des systèmes nerveux central et autonome des empoisonnements au sarin du métro de Tokyo. Pour évaluer les effets comportementaux et neurophysiologiques à long terme ou à retardement d'un empoisonnement au sarin, 9 hommes et 9 femmes qui ont été intoxiqués dans le métro de Tokyo le 20 mars 1995 ont été suivis pendant 6 à 8 mois par des tests neurologiques, une évaluation de stress post-traumatique ("post traumatic stress disorder", PTSD), des études de potentiel évoqué cérébral, une posturographie statique suivie par ordinateur, et une étude de la variabilité d'intervalle R-R électrocardiographique. Le jour de l'empoisonnement, l'activité de la cholinestérase sérique était de 13 à 131 UI/L (moyenne de 72,1 UI/L). Les résultats suggèrent que ces patients souffrent d'effets retardés sur leurs performances psychomotrices, leur système nerveux central et visuel et leur système vestibulaire, avec des symptômes psychiatriques révélés par le test de PTSD. (©Elsevier, Paris)

sarin / subway / Tokyo / neurobehavioral tests / posttraumatic stress disorder / computerized posturography / P300 / visual evoked potential / electrocardiographic R-R interval variability

1. Introduction

On June 27, 1994, about 600 residents in Matsuyama city, located approximately 150 km northwest of Tokyo, Japan, were poisoned due to a presumed terrorist attack with sarin (methyl phosphonofluoridic acid 1-methylethyl ester); all affected people recovered without abnormal findings on routine neurological and laboratory examinations except one patient who had severe anoxic encephalopathy due to respiratory arrest [24]. On March 20, 1995, a larger number of people (i.e., approximately 5500) were also poisoned with sarin in subways in Tokyo (Tokyo subway sarin poisoning). According to a follow-up study on the 640 cases, two cases died just after admission to hospital; no clinical abnormalities were found 3 months after the poisoning [29]. On the other hand, electroencephalogram (EEG) abnormalities in chemical plant workers have been reported more than 1 year after accidental exposure to sarin [10]. EEG changes in monkeys also have been observed 1 year after experimental sarin poisoning [7].

As a chronic neurological sequelae with decreased performance on neurobehavioral tests after acute or-

ganophosphate pesticide poisoning has been reported [30, 32, 37], we conducted a series of studies on delayed (long-term) effects of Tokyo subway sarin poisoning [27, 50, 51]. The paper presented here summarizes the results of our studies, indicating delayed neurobehavioral and neurophysiological effects of sarin poisoning together with posttraumatic stress disorder (PTSD).

2. Outline of the incidence

On March 20, 1995, 11 plastic bags containing odorous chemical agent were placed and poisonous vapors were released from the bags in five cars on three separate subway lines (Hibiya, Chiyoda and Marunouchi lines), which were scheduled to converge from the north and west on the Kasumigaseki Station (located in the government district) between 8:09 and 8:13 a.m. in Tokyo. As a result, approximately 5500 people were poisoned in the subway cars and stations at around 8:00 a.m.; nine passengers and two station officers died. The chemical agent was quickly identified as sarin by the Metropolitan Police Department and the Self-Defense Force.

The victims were transported to nearby hospitals to receive emergency medical evaluation and treatment. St. Luke's International Hospital, located within 3 km from the affected subway stations, accepted a total of 640 patients including the subjects examined in the present study, which was the largest number of the cases from the subway poisoning. The Metropolitan Police Department and the Tokyo District Public Prosecutors have suspected that the Aum Sinri Kyo cult released sarin at rush hour to create massive confusion in the Tokyo area, aiming at disturbing the police investigation into murder crimes including the poisoning in Matsumoto city [41].

3. Subjects

The nature of the procedure in the present study was fully explained to all subjects, and the study was conducted with their informed consent in September to November 1995, i.e., 6–8 months after the poisoning. Hospital staff could contact 150 victims of the poisoning and advise them to receive a health checkup on possible neurological sequelae during this period, nine males and nine females consented readily to undergo the study. Thus, a total of 18 patients (sarin cases) were examined: one plasterer, one kitchen maid and 16 office clerks, who had been exposed to sarin vapor accidentally in the subway attack in Tokyo on March 20, 1995, on the way to their work.

On admission to the hospital, plasma cholinesterase (ChE) activities ranged from 13 to 95 (mean 68.2) IU/L for females and from 19 to 131 (mean 75.9) IU/L for males (13 to 131 IU/L with a mean of 72.1 for all cases); all except for three males showed the values lower than 'normal' (100–250 IU/L). There was no significant difference in the ChE activities between female and male sarin cases ($P > 0.05$). Their symptoms and signs on admission are shown in *table I*. They were treated by injection of atropine and pralidoxim for 1–2 days. Age, level of education, amount and frequency of drinking, number of cigarettes smoked per day, past and present illness, and use of drugs were surveyed through an interview; amount of ethanol consumption per week was calculated using ethanol contents in beverages, i.e., 15% for sake, 43% for whiskey and 5% for beer.

Control subjects were three groups of healthy Japanese without history of occupational or environmental exposure to toxic chemicals including sarin, viz.: 1) eight males and seven females, whose age, male-female ratio, education level, alcohol consumption or smoking (cigarettes per day) were not significantly different from the sarin cases, for neurobehavioral tests; 2) 18 sex- and age-matched

Table I. Major symptoms and signs in 18 sarin cases on admission

	Number of cases		
	Male	Female	Total (%)
Dyspnea	5	6	11 (61)
Headache	5	6	11 (61)
Nausea	5	4	9 (50)
Feeling of asthenia	4	4	8 (44)
Diplopia	3	4	7 (39)
Ocular pain	3	4	7 (39)
Diarrhea	3		3 (17)
Sneeze	2	1	3 (17)
Sore throat	2	1	3 (17)
Paresthesia	2	1	3 (17)
Vomiting	1	2	3 (17)
Darkness of visual field	1	2	3 (17)
Decreased visual field	2		2 (11)
Anxiety	2		2 (11)
Cough	1	1	2 (11)
Loss of consciousness	1	1	2 (11)
Cardiac arrest		1	1 (6)
Lacrimation		1	1 (6)

controls without significant differences in age, alcohol consumption or smoking from the cases for brain evoked potentials and electrocardiographic R-R interval variability (CVRR); and 3) 18 females and 35 males, whose age, height, body weight, alcohol consumption or smoking were not significantly different from the cases, for posturography.

4. Methods

4.1. Neurobehavioral test

Subjects underwent nine tests: 1) digit symbol (psychomotor performance); 2) picture completion (visual perception); 3) digit span (attention and memory); 4) finger tapping (psychomotor performance); 5) reaction time (psychomotor performance); 6) continuous performance test (sustained visual attention); 7) paired-associate learning (learning and memory); 8) General Health Questionnaires (GHQ) (psychiatric symptoms); and 9) Profile of Mood States (POMS) (mood), in a quiet room during day time of weekdays. The first three tests (1–3) are subtests of the Japanese edition of the Wechsler Adult Intelligence Scale (WAIS) [17]; the second three (4–6) are subtests of Japanese version of computer-administered testing (Neurobehavioral Evaluation System) [19, 45]; test 7 is a subtest of the Japanese version of Wechsler Memory Scale (WMS) [15, 43]; and the last two (8, 9) were self-rating questionnaires [13, 16, 23, 46, 47].

4.2. Posttraumatic stress disorder (PTSD) checklist

A self-rating questionnaire consisting of 17 problems and complaints related to PTSD (*Appendix*) was administered on all subjects just before neuro-behavioral testing. This was originally developed for PTSD based on DSM-III-R in veterans with military experience [42]; in the present study the list was translated into Japanese in which the term 'military experience' in the original form was substituted by subway sarin incident. The subject was asked to indicate how much he (she) had been bothered by that problem or complaints in the past month, using a rating from 1 (not at all) to 5 (extremely); the sum of the numbers was used as the score on PTSD.

4.3. Brain evoked potentials

The event-related evoked potentials (ERP) were measured as described previously [2, 3, 12]. A random sequence of two distinguishable tones (90 dB SPL) was presented binaurally through earphones at a rate of 0.5 Hz. Eighty percent of the 320 tones had a frequency of 1000 Hz (non-target tones) and 20% had a frequency of 2000 Hz (target tones). The subject was instructed to count the target tone mentally. In all cases, the reported total of the target tone was correct within three stimuli of the actual tones presented. Cerebral responses to the two stimuli were recorded using disc electrodes at the vertex, mastoids and forehead, and were averaged separately. The P300 component elicited by the target tone was defined as the first maximal positive wave between 250 and 500 ms; the N100 component by the non-target tone was also recorded to examine if the change in P300 was independent of the early auditory responses.

The pattern-reversal visual evoked-potential (VEP) was measured in a darkened room [1, 3, 36]. The subject sat in front of TV screen and was given a checkerboard pattern on the screen consisting of white and black squares, reversing at a rate of 2 Hz. One positive peak (P100) was recorded using standard EEG electrodes fixed to the occipital cortex, forehead and left mastoid (grand). Responses were averaged over 128 times.

The brainstem auditory evoked potential (BAEP) was measured by the method reported previously [3, 38]. Click signals were presented to the right ear through earphones at rates of 20 Hz. The BAEP was recorded using three standard EEG electrodes on the vertex, right mastoid and left mastoid (grand). The responses were averaged 1024 times with one replication.

4.4. Computerized static posturography

Postural balance was quantitatively measured by the method reported previously by us [4, 5, 48–50]. Measurements were carried out in a quiet room with flat floor using a strain-gauge-type force platform (Static Sensograph 1G06, NEC Sanei, Tokyo) connected to a microcomputer (PC9801, NEC, Tokyo) via an analog-to-digital converter (Analog Pro, Canopus, Tokyo). Subjects were instructed to refrain from alcohol or drugs the day before the study (12 h or more prior to the study).

Subjects were asked to stand quietly on the platform for 60 s with eyes open and then for 60 s with eyes closed. Strain gauges at the three corners of the platform measured vertical forces and converted them to electric voltages; the medio-lateral (right-left) and anterior-posterior location of the body's center of pressure (CP) in the horizontal plane was calculated from the voltages sampled at a frequency of 20 Hz by the microcomputer and recorded on a disk.

Displacements of the body's CP in the both directions were then subjected into the Fast Fourier Transformation analysis [5, 9, 48–50]. The total duration of sampling was 51.2 s (1024 points). The span of frequencies analyzed ranged from 0 to 4 Hz (0.02-Hz steps). Sum of the root squares of the power calculated from the power spectrum between: 1) 0 and 1 Hz; 2) 1 and 2 Hz; and 3) 2 and 4 Hz served as measures of the amount of postural sway for each frequency range with eyes open and closed.

Lengths of displacement of the CP in the medio-lateral and anterior-posterior directions within each sampling time were summed up for each direction and defined as the length of sway path of the body's CP in the medio-lateral (Dx) and anterior-posterior (Dy) directions, respectively; the area included within the sway path traveled by the CP during 60 s (Area) was also calculated. Romberg quotients, i.e., the ratio of the value with eyes closed to that with eyes open [5, 9, 48–50], for the power of the sway for each frequency band, Dx, Dy and Area, were calculated.

Some studies [6, 8, 9, 11, 21, 44] have suggested that: 1) the lesion of the vestibulo-cerebellum (lower vermis), which contains the vestibulo-cerebellar pathway, shows a sway of non-specific frequencies with eyes open (low Romberg quotient); 2) the lesion of the anterior cerebellar lobe is related to a sway of high frequency (2–4 Hz) predominantly in anterior-posterior direction with eyes closed (high Romberg quotient); and 3) the lesion in the spinocerebellar afferent pathway (including the spinocerebellar tract and proprioceptive nerve fibers in the legs) is associated with an omnidirectional sway

of low frequency (1 Hz or less) with eyes closed (high Romberg quotient).

4.5. Electrocardiographic R-R interval variability

Three-hundred R-R intervals on electrocardiogram were measured after the subject had been supine for 10 min; 100 consecutive R-R intervals with the minimal standard deviation (S.D.) were extracted [5, 25, 26]. The CVRR was defined as the ratio of S.D. to the mean (%). The spectrum of R-R intervals was calculated by autoregressive spectral analysis, and separated for the low frequency (LF) at the center frequency of 0.04–0.15 Hz and the high frequency (HF) component at 0.15–0.4 Hz by component analysis. Each component coefficient of variation (C-CVLF and C-CVHF, %) was defined as the ratio of the square root of each component to the mean R-R interval. The C-CVLF and C-CVHF are thought to reflect sympathetic and parasympathetic activities, respectively [40].

5. Results

The score on digit symbol test in the sarin cases was significantly lower than that in the controls; the scores on GHQ, fatigue (POMS) and PTSD checklist were significantly higher in the former group than

in the latter (table II). When the score on PTSD was added to the covariates, only the score on the digit symbol test in the sarin cases was significantly lower than in the controls (table II). Scores on GHQ and fatigue (POMS) were significantly correlated with PTSD scores in 18 sarin cases ($r = 0.747$ and 0.471 , respectively, $P < 0.05$). The P300 (ERP) and P100 (VEP) latencies in the sarin cases were significantly prolonged when compared with the controls (table II). In females, the power of postural sway of 0–1 Hz in the sarin cases was significantly larger than that in the controls in the anterior-posterior direction when eyes were open (table II); similarly, the Area with eyes open was significantly larger in these cases (table II). The Romberg quotient for the power of 0–1 Hz of sway in the anterior-posterior direction was significantly correlated with log ChE in the females ($r = 0.673$, $P < 0.05$).

No other measures in the present study showed significant differences between the cases and controls ($P > 0.05$).

6. Discussion

A significant decrease in the score on digit symbol test was observed in the cases 6–8 months after acute sarin poisoning. This decrease was also significant when the effect of PTSD was controlled in the analysis

Table II. Significant differences in neurobehavioral tests, posttraumatic stress disorder (PTSD) checklist, brain evoked potentials and computerized posturography between sarin cases and controls.

	Cases		Controls		Significance level (P)
	Mean	Range	Mean	Range	
<i>Neurobehavioral tests^a</i>					
Digit symbol	15.9	11–19	17.5	14–19	< 0.05 ^b
General Health Questionnaires	5.6	0–13	2.5	0–9	< 0.01 ^c
Profile of Mood States (fatigue)	11.6	2–23	6.7	0–17	< 0.05 ^c
PTSD ^a	25.9	18–45	21.2	17–38	< 0.05
<i>Brain evoked potentials (ms)^d</i>					
P300 (event-related potential)	321	284–348	289	248–325	< 0.001
P100 (visual-evoked potential)	102	91–114	97	82–118	< 0.05
<i>Posturography (eyes open)^e</i>					
Power of 0–1 Hz sway, anterior-posterior direction (cm)	3.47	2.1–4.9	2.72	1.7–3.4	< 0.05
Area (cm ²)	2.74	1.1–5.0	2.12	0.7–3.2	< 0.05

^a18 sarin cases and 15 controls; analysis of covariance where factors were group (sarin case or control) and gender and covariates were age, education level, alcohol consumption and smoking.

^b $P < 0.01$ when PTSD scores were added to covariates.

^c $P > 0.05$ when PTSD scores were added to covariates.

^d18 sarin cases and the same number of age- and sex-matched controls; paired *t*-test.

^eNine females sarin cases and 18 female controls; analysis of covariance where factor was group (sarin case or control) and covariates were age, height, body weight and alcohol consumption.

of covariance. It is thus suggested that a chronic effect on psychomotor performance (motor persistence, sustained attention, response speed, and visuomotor coordination) as measured by digit symbol test was caused directly by acute sarin poisoning. Similarly, the P300 (ERP) and P100 (VEP), which are considered to reflect cognitive function and the conduction time from the retinal to the visual cortex, respectively [12, 38], were prolonged in the sarin cases, indicating the delayed effects of sarin on the higher and visual system. These findings essentially coincide with the observations of chronic neurological sequelae after acute organophosphate poisoning including sarin [7, 10, 30, 32, 37].

In the posturography, the sway of low (0–1 Hz) frequency and the area of sway with eyes open were significantly increased in the female sarin cases; the Romberg quotient for the former measure was significantly related to log ChE in the cases. Therefore, it is suggested that a delayed (long-term) effect on the vestibulo-cerebellar type of sway was caused in females. This may coincide with the observations of neurological sequelae after acute organophosphate poisoning. However, in contrast to the present study, pesticide applicators with concurrent exposure to organophosphate showed that a total length of the sway path was affected more with eyes closed than with eyes open [31]; the discrepancy between the two studies remains unexplained. The result that the posturographic effects were observed only in females in the present study agrees with the finding that the effects of organophosphate (maloxon) are more severe in female than in male rats [33].

The posturographic change in females was significantly related to log ChE, agreeing with the findings that acetylcholine mediates the vestibulo-cerebellar function [20, 34]. In contrast, the relationship of the score on digit symbol test, P300 or P100 to ChE was not significant. This may coincide with the observations in rats that ChE in the brain just after poisoning by sarin did not predict signs of encephalopathy 1–2 days after the poisoning [18] and that behavioral abnormalities were not correlated with concurrent brain acetylcholinesterase activities 6 and 24 h after poisoning by sarin, soman or tabun (N-dimethylphosphoramidate-cyanidate, a toxic organophosphate compound) [14]. Thus, the mechanism of the central nervous system toxicity of sarin and related organophosphate compounds other than the inhibition of acetylcholinesterase activity should be investigated.

Lesions in the brain, i.e., neuronal degeneration and necrosis, have been observed in rats surviving single subcutaneous injection of sarin or soman (O-(1,2,2-trimethylpropyl)-methylfluorophosphate, an organophosphate compound with potential

for use in war) [18, 22, 35]. One of these studies [35] examined the histopathological changes up to 35 days after exposure and revealed that the changes were still present, suggesting that toxic organophosphate compounds such as sarin and soman can produce irreversible (or long-term) damage to the central nervous system. It is, therefore, possible that the significantly reduced scores on the digit symbol test in the cases in the present study can be attributed to irreversible changes in the central nervous system caused by sarin.

The score on PTSD in the sarin cases was significantly higher than in controls, agreeing with the observation of PTSD in the cases of Tokyo subway sarin poisoning [28, 29]. When PTSD score was not used as a covariate in the analysis of covariance, the scores on GHQ and fatigue (POMS) were significantly increased in the cases whereas the increases were not significant after PTSD was added to the covariates. Also, the scores on GHQ and fatigue were positively related to PTSD in sarin cases. Therefore, psychiatric (GHQ) and mood (POMS) changes are considered to result from PTSD. As psychological symptoms, such as complaints of physical distress and psychopathological profiles of Minnesota Multiphasic Personality Inventory, related to PTSD have been observed in Korean war veterans 30 years after the war [39], the psychiatric and mood changes might have had persisted in the cases since the Tokyo subway sarin poisoning.

Finally, because of small sample size and low recruitment of sarin cases in the present study, a further study on a larger number of victims of the Tokyo subway sarin poisoning will be necessary to confirm the observations of chronic neurobehavioral effects of sarin and of PTSD. Also, imaging techniques such as magnetic resonance imaging may be useful for examining structural changes in brain to related to sarin exposure and/or PTSD.

Appendix

PTSD checklist

1. Repeated, disturbing memories of the subway sarin incident?
2. Repeated, disturbing dreams of the subway sarin incident?
3. Suddenly acting or feeling as if the subway sarin incident happens again?
4. Feeling very upset when something happened that reminded you of the subway sarin incident?
5. Trouble remembering important parts of the subway sarin incident?
6. Loss of you interest in activities that you used to enjoy?

7. Feeling distant or cut off from other people?
8. Feeling emotionally numb, or being unable to have loving feelings for those close to you?
9. Feeling as if your future will somehow be cut short?
10. Trouble falling or staying asleep?
11. Feeling irritable or having angry outbursts?
12. Having difficulty concentrating?
13. Being 'superalert', or watchful or on guard?
14. Feeling jumpy or easily startled?
15. Having physical reactions when something reminds you of the subway sarin incident?
16. Avoid thinking about the subway sarin incident, or avoid having feeling about it?
17. Avoid activities or situations because they reminded you of the subway sarin incident?

Acknowledgment

The authors thank Dr. Roberta F. White, Department of Neurology, Boston University School of Medicine for her valuable suggestions.

References

- [1] Araki S., Murata K., Aono H., Central and peripheral nervous system dysfunction in workers exposed to lead, zinc and copper: a follow-up study of visual and somatosensory evoked potentials, *Int. Arch. Occup. Environ. Health* 59 (1987) 177-187.
- [2] Araki S., Murata K., Yokoyama K., Uchida E., Auditory event-related potential (P300) in relation to peripheral nerve conduction in workers exposed to lead, zinc and copper: effects of lead on cognitive function and central nervous system, *Am. J. Ind. Med.* 21 (1992) 539-547.
- [3] Araki S., Murata K., Determination of evoked potentials in occupational and environmental medicine: a review, *Environ. Res.* 63 (1993) 133-147.
- [4] Araki S., Murata K., Yokoyama K., Application of neurophysiological methods in occupational medicine in relation to psychological performance, *Ann. Acad. Med. Singapore* 23 (1994) 710-718.
- [5] Araki S., Yokoyama K., Murata K., Neurophysiological methods in occupational and environmental health: methodology and recent findings, *Environ. Res.* 73 (1997) 42-51.
- [6] Asahina M., Nakajima M., Kosjima S., Hirayama K., Postural sway in patients with hereditary ataxia, *Rinsho Shinkeigaku (Clin. Neurol.)* 34 (1994) 1105-1110 (in Japanese with English abstract).
- [7] Burchfield J.L., Duffy F.H., Sim V.M., Persistent effects of sarin and diethylrin upon the primate electroencephalogram, *Toxicol. Appl. Pharmacol.* 35 (1976) 365-379.
- [8] Dickgans J., Fetter M., Computerized cerebellar functions upon the stabilization of body posture, *Rev. Neurol.* 149 (1993) 654-664.
- [9] Diener H.C., Dichgans J., Bacher M., Gompf B., Quantification of postural sway in normals and patients with cerebellar diseases, *Electroencephal. Clin. Neurophysiol.* 1984 (1984) 57,134-142.
- [10] Duffy F.H., Burchfield J.L., Bartels P.H., Gaon M., Sim V.M., Long-term effects of an organophosphate upon human electroencephalogram, *Toxicol. Appl. Pharmacol.* 47 (1979) 161-176.
- [11] Fitzpatrick R.C., Gorman R.B., Burke D., Gandevia S.C., Postural proprioceptive reflexes in standing human subjects: bandwidth of response and transmission characteristics, *J. Physiol.* 458 (1992) 69-83.
- [12] Goodin D.S., Event-related (endogenous) potentials, in: Aminoff M.J. (Ed.), *Electrodiagnosis in Clinical Neurology*, Churchill Livingstone, New York, 1986, pp. 575-595.
- [13] Goldberg D.P., The Detection of Psychiatric Illness by Questionnaire: A Technique for the Identification and Assessment of Non-psychotic Psychiatric Illness, Oxford University Press, London, 1972.
- [14] Hoskins B., Fernando J.C.R., Dulaney M.D., Lim D.K., Liu D.D., Watanabe H.K., Ho I.K., Relationship between the neurotoxicities of soman, sarin and tabun, and acetylcholinesterase inhibition, *Toxicol. Lett.* 36 (1986) 121-129.
- [15] Kiba K., Nakamura M., Hiramatsu H., Yamaguchi N., Kurachi M., A study of Japanese version of Wechsler Memory Scale: a comparison on scores of schizophrenics and normals, *Psychiatr. Med. (Seishin Igaku)* 30 (1988) 635-642.
- [16] Kitamura T., Sugawara M., Aoki M., Shima S., Validity of the Japanese version of the GHQ among antenatal clinic attendants, *Psychol. Med.* 19 (1989) 507-511.
- [17] Kodama H., Shinagawa F., Indo T., WAIS Japanese Edition, Nihon Bunka Kagakusha, Tokyo (in Japanese), 1982.
- [18] Lemerrier G., Carpentier P., Sentenac-Roumanou H., Morelis P., Histological and histochemical changes in the central nervous system of the rat poisoned by an irreversible anticholinesterase organophosphorus compound, *Acta Neuropathol.* 61, (1983) 123-129.
- [19] Letz R., Baker E.L., Computer-administered neurobehavioral testing in occupational health, *Semin. Occup. Med.* 1 (1986) 197-203.
- [20] Matsuoka M., Ito J., Takahashi H., Sasa M., Takaori S., Experimental vestibular pharmacology: a minireview with special reference to neuroactive substances and antivergo drugs, *Acta Otolaryn.* 419 (Suppl.) (1985) 62-70.
- [21] Mauritz K.H., Dichgans J., Hufschmidt A., Quantitative analysis of stance in late cortical cerebellar atrophy of the anterior lobe and other forms of cerebellar ataxia, *Brain* 102 (1979) 461-482.
- [22] McLeod C.G., Singer A.W., Harrington D.G., Acute neuropathology in soman poisoned rats, *Neurotoxicology* 5 (1984) 53-58.
- [23] McNair D.M., Lorr M., Droppelman L.F., Profile of Mood States, Educational and Industrial Testing Service, San Diego, 1992.
- [24] Morita H., Yanagisawa N., Nakajima T., Shimizu M., Hirabayashi H., Okudera H., Nohara M., Midorikawa Y., Mimura S., Sarin poisoning in Matsumoto, *Lancet* 346 (1995) 290-293.
- [25] Murata K., Araki S., Yokoyama K., Nomiyama H., Tao Y.X., Liu S.J., Autonomic and central nervous system effects of lead in female glass workers in China, *Am. J. Ind. Med.* 28 (1995) 233-244.
- [26] Murata K., Araki S., Assessment of autonomic neurotoxicity in occupational and environmental health as determined by ECG R-R variability: a review, *Am. J. Ind. Med.* 30 (1996) 155-163.
- [27] Murata K., Araki S., Yokoyama K., Okumura T., Ishimatsu S., Takasu N., White R.F., Asymptomatic sequelae to acute

- sarin poisoning in the central and autonomic nervous system 6 months after the Tokyo subway attack, *J. Neurol.* 244 (1997) 601–606.
- [28] Nakao M., Kawana N., Report from psychiatric department in meeting on treatment of sarin poisoning patients at St. Luke's International Hospital, *Jpn. Med. J.* 3706 (1995) 55–56 (in Japanese).
- [29] Okumura T., Nobukatsu T., Ishimatsu S., Miyanoki S., Mitsuhashi A., Kumda K., Tanaka T., Hinohara S., Report on 640 victims of the Tokyo subway sarin attack, *Ann. Emerg. Med.* 28 (1996) 129–135.
- [30] Rosenstock L., Keifer M., Daniel W.E., McConnell R., Claypoole K., Chronic central nervous system effects of acute organophosphate pesticide intoxication, *Lancet* 338 (1991) 223–227.
- [31] Sack D., Linz D., Shulka R., Rice C., Bhattacharya A., Suskind R., Health status of pesticide applicators: postural stability assessments, *JOM* 35 (1993) 1196–1202.
- [32] Savage E.P., Keefe T.J., Mounce L.M., Heaton R.K., Lewis J.A., Burcar P.J., Chronic neurological sequelae of acute organophosphate pesticide poisoning, *Arch. Environ. Health* 43 (1988) 38–45.
- [33] Savolainen K.M., Hiroven M.-R., Second messengers in cholinergic-induced convulsion and neuronal injury, *Toxicol. Lett.* 64/65 (1992) 437–445.
- [34] Silver A., Cholinesterases of the central nervous system with special reference to the cerebellum, *Int. Rev. Neurobiol.* 10 (1967) 57–109.
- [35] Singer A.W., Jaax N.K., Graham J.S., McLeod C.G., Cardiomyopathy in soman and sarin intoxicated rats, *Toxicol. Lett.* 36 (1987) 243–249.
- [36] Sokol S.A., Visual evoked potentials, in: Aminoff M.J. (Ed.), *Electrodiagnosis in Clinical Neurology*, Churchill Livingstone, New York, 1986, pp. 441–466.
- [37] Steenland K., Jenkins B., Ames R.G., O'Malley M., Chrislip D., Russo J., Chronic neurological sequelae to organophosphate pesticide poisoning, *Am. J. Pub. Health* 84 (1994) 731–736.
- [38] Stockard J.J., Stockard J.E., Sharbrough F.W., Brainstem auditory evoked potentials in neurology: methodology, interpretation, and clinical application event-related (endogenous) potentials, in: Aminoff M.J. (Ed.), *Electrodiagnosis in Clinical Neurology*, Churchill Livingstone, New York, 1986, pp. 467–503.
- [39] Sutker P.B., Winstead D.K., Galina Z.H., Allain A.N., Cognitive and psychopathology among former prisoners of war and combat veterans of the Korean conflict, *Am. J. Psychiatry* 148 (1991) 67–72.
- [40] Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, Heart rate variability: standards of measurement, physiological interpretation, and clinical use, *Circulation* 93 (1996) 1043–1065.
- [41] Tokyo District Public Prosecutors, Opening Statement in the Trial of Shoko Asahara, the Tokyo District Court, April 25, 1996. Adopted from Yamanashi Nichi Nichi Shinbun (newspaper) April 26, 1996, 21–23 (in Japanese).
- [42] Weathers F.W., Litz B.T., Herman D.S., Huskin J.A., Keane T.M., The PTSD check list (PCL): Reliability, validity, and diagnostic utility. In: *Proceedings of the 9th Annual Meeting of International Society for Traumatic Stress Studies (Trauma, Coping, and Adaptation)*, Texas, Oct 24–27, 1993.
- [43] Wechsler D., A standardized memory scale for clinical use, *J. Psychol.* 19 (1945) 87–95.
- [44] Yagi K., Multivariate statistical analysis in stabilometry in human upright standing (the first report): age-related factor, *Jpn. J. Otorhinolaryngol.* 92 (1989) 899–908 (in Japanese with English abstract).
- [45] Yokoyama K., Araki S., Osuga J., Karita T., Kurokawa M., Koda K., Development of Japanese edition of Neurobehavioral Evaluation System (NES) and WHO Neurobehavioral Core Test Battery (NCTB): with assessment of reliability, *Jpn. J. Ind. Health* 32 (1990) 334–355 (in Japanese).
- [46] Yokoyama K., Araki S., Kawakami N., Takeshita T., Production of Japanese edition of Profile of Mood States (POMS): Assessment of reliability and validity, *Jpn. J. Pub. Health* 37 (1990) 913–918 (in Japanese with English abstract).
- [47] Yokoyama K., Araki S., Manual for Japanese Edition of Profile of Mood States (POMS), Kaneko-shobo, Tokyo, 1994 (in Japanese).
- [48] Yokoyama K., Araki S., Murata K., Morita Y., Katsuno N., Tanigawa T., Mori N., Yokota J., Ito A., Sakata E., Subclinical vestibulo-cerebellar, anterior cerebellar lobe and spinocerebellar effects in lead workers in relation to concurrent and past exposure, *Neurotoxicology* 18 (1997) 371–380.
- [49] Yokoyama K., Araki S., Murata K., Nishikitani M., Nakaaki K., Yokota J., Ito A., Sakata E., Postural sway frequency analysis in workers exposed to *n*-hexane, xylene, and toluene: assessment of subclinical cerebellar dysfunction, *Environ. Res.* 74 (1997) 110–115.
- [50] Yokoyama K., Araki S., Murata K., Nishikitani M., Okumura T., Ishimatsu S., Takasu N., A preliminary study on delayed vestibulo-cerebellar effects of Tokyo Subway Sarin Poisoning in relation to gender difference: frequency analysis of postural sway, *JOEM* 40 (1998) 17–21.
- [51] Yokoyama K., Araki S., Murata K., Nishikitani M., Okumura T., Ishimatsu S., Takasu N., White R.F., Chronic neurobehavioral effects of Tokyo Subway Sarin Poisoning in relation to posttraumatic stress disorder, *Arch. Environ. Health* 53 (1998), in press.

Long term health effects of low dose exposure to nerve agent

David H. Moore

Battelle Edgewood Operations, 2012 Tollgate Road, Suite 206, Bel Air, MD 21015, USA

Abstract — Possible long-term toxic effects of nerve agents have been investigated using sensitive toxicological screens and extensive toxicity studies in various animal models. Data on humans have been obtained from controlled studies and accidental exposures. Studies in the area of 'low dose' exposure to nerve agents are currently being performed. (©Elsevier, Paris)

Résumé — Effets à long terme de faibles doses d'agents neurotoxiques. Les éventuels effets toxiques à long terme d'agents neurotoxiques ont été analysés grâce à des tests toxicologiques sensibles, et à des études de toxicité dans divers modèles animaux. Les données concernant les humains ont été obtenues à partir d'études expérimentales contrôlées et d'intoxications accidentelles. L'étude de faibles doses d'agents neurotoxiques est actuellement en cours. (©Elsevier, Paris)

nerve agent / soman / sarin / tabun / VX / acetylcholinesterase / EEG / delayed neurotoxicity / subchronic

1. Introduction

Nerve agents exert their effects by inhibition of the enzyme acetylcholinesterase (AChE), leading to accumulation of excess levels of the neurotransmitter acetylcholine (ACh) at cholinergic synapses. Enzyme inhibition is both rapid and irreversible, thus making organophosphorus (OP) nerve agents such as tabun (GA), sarin (GB), soman (GD), cyclosarin (GF) and VX highly toxic and extremely dangerous chemicals designed to kill or incapacitate enemy forces, disrupt military operations and deny terrain to the adversary. Additionally, nerve agents have been used recently as weapons of terror. In June 1994 and again in March 1995, sarin was released in public places by a Japanese cult. In the latter incident, 5500 people on Tokyo subways sought medical care; about 4000 suffered no effects from the agent, but 12 people died [1].

Nerve agents gain entry by absorption through the lungs or skin and impair the activity of cholinergic synapses, including those of smooth and skeletal muscle, autonomic ganglia and the central nervous system (CNS). Acute toxic effects of nerve agents can be elicited at very low concentrations while lethal effects are observed at somewhat higher concentrations. Threshold symptoms for vapor exposure are commonly stated to be miosis, rhinorrhea and airway constriction, generally appearing at a Ct (vapor concentration \times exposure time) of 2–3 mg/min/m³ [2]. Low to moderate exposure of skin to liquid nerve agent causes localized sweating, nausea, vomiting and a feeling of weakness [3]. Lethal amounts of vapor or liquid cause a rapid cascade of events culminating within a minute or two in convulsions, loss of consciousness, apnea, flaccid

paralysis and death [3]. Toxicity is thus concentration-dependent, requiring a defined minimal concentration of agent; recovery generally occurs by synthesis of new AChE (See *tables I and II* for toxicity of nerve agents).

Concerns regarding Persian Gulf Illness as well as ongoing concerns about low-level chemical warfare exposures and potential exposures to low-levels of sarin at Khamisiyah, Iraq in March, 1991 have led to much discussion about the consequences of low dose exposure to nerve agent, especially long term effects of such exposure on human health.

2. Low-level nerve agent exposure and long term health effects

Low levels of exposure to nerve agent can be considered as those levels that result in minimal reduc-

Table I. Vapor toxicity (mg/min/m³).

Agent	LCt ₅₀	ECt ₅₀ (miosis)
GA	400	2–3
GB	100	3
GD	70	< 1
GF	UNK	< 1
VX	50	0.04

Table II. LD₅₀ on skin.

Agent	Amount (mg)
GA	1000
GB	1700
GD	50
GF	30
VX	10

tion of AChE with no observable clinical signs and no subjective symptoms. Three levels of low dose exposure can be described: level 1 is an exposure that results in no clinical signs or symptoms and an AChE inhibition of < 20%; level 2 is an exposure that results in no clinical signs or symptoms but a moderate inhibition of AChE (> 20%); and level 3 is an exposure that results in mild clinical signs such as salivation, miosis and tachycardia and subjective symptoms such as shortness of breath. Exposure values in humans have been established for GB (*table III*) and are explained below.

The LC₅₀ is the dose expected to kill 50% of the exposed population. No neuromuscular effects (NNM) are expected at doses below 4 mg min/m³. No observable effects (NOEL) are seen with levels at or below 0.5 mg min/m³. An 8-h exposure to 0.05 mg min/m³ will remain below NOEL. The remaining values in *table III* represent occupational exposure limits as established by the U.S. Department of Health and Human Services. Significant safety factors have been applied.

Table III. Human exposure values for GB.

Agent GB
LC ₅₀ = 100 mg min/m ³
No death dose = 10 mg min/m ³
NNM effect dose = 4 mg min/m ³
EC ₅₀ (miosis) = 2–4 mg min/m ³
NOEL = 0.5 mg min/m ³
MSC (1 h) = 0.001 mg/m ³
MSC (8 h) = 0.0003 mg/m ³
Safety factor of 0.1 for general population
0.0001 mg/m ³ (1 h)
0.00003 mg/m ³ (8 h)
0.000003 mg/m ³ (72 h)

Data available on humans exposed to nerve agents comes from two sources: human experiments and accidental exposures.

2.1. Controlled studies on volunteers

Between 1958 and 1975, the U.S. Army conducted research involving 6000 soldier volunteers to learn how specific chemicals may affect humans. Of this group, over eleven hundred subjects were exposed to low and moderate levels of nerve agents under controlled conditions. The doses used in these experiments were equal to or less than 1.5 ID₅₀ units. The ID₅₀ is the dose of agent that causes early signs of incapacitation (miosis) in 50% of exposed individuals. In 1982, the U.S. Army requested that the Committee on Toxicology of the National Research Council conduct a study of the possible chronic ad-

verse health effects on the above group of servicemen who had been exposed to chemical agents under experimental conditions. The first report found no evidence to support a finding of adverse long-term or delayed health effects following exposure to nerve agents [4]. However, this report was unable to rule out the possibility that some anti-AChE agents produced long-term adverse health effects in some individuals. The report deferred to the outcome of a follow-on morbidity study to 'shed further light' on this issue. In the follow-on study, a questionnaire was sent to subjects of the earlier studies in order to assess the current health status of over 4000 subjects voluntarily exposed to chemical agents between 1958–1975 [5]. The long-term health effects of greatest interest included: 1) increased cancer risk; and 2) adverse mental, neurological, hepatic and reproductive effects. Results indicated that subjects who received nerve agents, as a group, did not differ from controls who had received no chemical treatment.

2.2. Accidental exposures and electroencephalographic changes

Over the past 50 years, hundreds of industrial and laboratory workers have been accidentally exposed to both asymptomatic and symptomatic levels of nerve agents. The strongest evidence in humans of a possible long-term effect of exposure to nerve agents is from studies reporting changes in electroencephalograms (EEGs). There is no single study or set of studies that exactly addresses the acute and long-term changes in EEG activity produced by nerve agents. Also, virtually all the animal EEG studies of nerve agent exposure have focused on the effects of high dose exposure and the mechanisms and treatment of these more serious toxic effects. Estimated ranges of red blood cell cholinesterase inhibition are correlated to observed EEG effects.

Chronologically, the first study to suggest long-term EEG effects following nerve agent exposures was by Metcalf and Holmes, who described their findings from a group of workers at a sarin production plant [6]. Exposed workers had higher voltage EEGs with more pronounced alpha rhythm and bursts of slow waves during drowsiness. The individuals also had a high incidence of 'narcoleptic' sleep patterns, corresponding presumably to early REM sleep.

The widely cited work of Duffy et al. [7] describes EEG changes found in a group of 77 workers accidentally exposed to sarin showing signs and symptoms of exposure and AChE inhibition > 25%. Forty-one of the 77 workers had multiple (> 3) exposures. EEGs from this group were compared to those of a control group from the general population. Subtle, but statistically significant increases in EEG

beta-band activity over controls were observed for 1 to 6 years after exposure in the sarin group. Individuals exposed to sarin were reported to have more and longer periods of REM sleep than the general population. This was also true, however, of the control group in this study. These 77 workers reported no adverse health effects and no behavioral changes. The significance of the alterations in EEG patterns is uncertain because no behavioral effects can be attributed to them. The panel analyzing the results from the U.S. Army study [5] could neither confirm nor exclude the findings of Duffy et al. [7]. To summarize what is presently known, long-term changes in EEGs have been observed with symptomatic exposures; multiple exposures produce more prominent effects. Increases in beta activity are seen during drowsiness or hyperventilation. Increases in REM sleep occur after OP exposure. Records are also available on workers with exposures to OP pesticides [8].

2.3. Animal studies using primates

Results are available from a non-human primate study using sarin [9]. Rhesus monkeys were implanted with cortical and depth electrodes and injected with sarin in one of two dosage schedules: a single high dose (5 µg/kg, i.v.) that elicited seizures, or low doses (1 µg/kg, i.m.) once a week for 10 weeks that caused no clinical effects. EEGs were recorded prior to exposure, 24 h and 1 year after exposure. The animals from both dose schedules had increases in high frequency beta activity, but were otherwise healthy. No long-term behavioral effects were noted.

2.4. Other animal studies

There are few data on asymptomatic doses in animals since high doses are generally selected by investigators to elicit observable effects. Studies have been performed using chronic exposures to symptomatic non-lethal doses of OP nerve agents (GA, GB, GD and VX). With the exception of one study using GA, the seven studies reported no persistent changes in histopathology, hematology, clinical chemistry or other biochemical parameters [10]. Another group of five studies used subchronic, symptomatic doses of GD, GB or VX up to two times the ID₅₀. Myopathy was consistently produced with cardiomyopathy prominent in animals experiencing seizures [11].

Attenuation of hormonal responses to physiological or pharmacological challenge was observed in a single study 2 weeks after an acute symptomatic dose of GD, possibly attributable to suppression of diurnal hormonal cycles [12].

2.5. Toxicological studies

The majority of closely controlled toxicological studies have been conducted in animals at the National Center for Toxicological Research or the University of California, Davis following strict guidelines. The G-agents have been screened for mutagenicity or clastogenicity using *in vitro* and *in vivo* assays. GB and GD have been found not to be mutagenic [13]. GA was found to be weakly mutagenic in three different assays but not teratogenic in two animal species, rabbits and rats [14]. Essentially, investigation of long-term toxic effects of nerve agents using sensitive toxicological screens and extensive toxicity studies in various animal models has yielded negative results over a wide range of parameters.

2.6. Delayed neurotoxicity

Evidence for neurotoxicity and neuropathology has been sought in a number of studies using nerve agents. Since it is widely known that certain OP pesticides produce a delayed neuropathy, nerve agents have been tested for the same phenomenon: OP-induced delayed neurotoxicity (OPIDN), using the white leghorn hen, an animal model sensitive to delayed neurotoxicity. Wide ranging doses of nerve agents were employed in 11 studies: no neuropathy or neurotoxic esterase inhibition was seen with VX or with G-agents at doses < 11 LD₅₀ in animals treated with atropine and pralidoxime chloride to ensure survival [15]. According to Sidell and Hurst [16]: 'the syndrome (delayed neurotoxicity) has not been noted in the handful of humans severely exposed to nerve agents or in the hundreds of humans with mild to moderate effects from nerve agents.' Thus, in this respect, nerve agents differ significantly from some OP pesticides.

OP pesticides and OP nerve agents have much in common, but differ significantly in their acute toxicity and physical properties. Some of their targets and some effects also differ. Nerve agents do not produce OPIDN at concentrations achievable with vapor exposure. Therefore, extrapolation from pesticide effects as they relate to neurotoxicity may be inappropriate.

3. Conclusion

Controlled studies of human exposures, reports of accidental exposures, and animal studies collectively indicate that exposures to low level OP nerve agents do not produce chronic illness. None of the nerve agents have been shown to be mutagenic or carcinogenic. Nerve degeneration is considered an unlik-

ely outcome either from acute or long-term exposure to any nerve agent. Long-term EEG changes are known, but they are of uncertain significance. The majority of the data supports the conclusion that there are no observable long-term adverse health effects following exposure to asymptomatic levels of nerve agents. In fact, the final recommendation of the Department of Health and Human Services, published in the Federal Register on March 15, 1988 stated: 'Questions related to the nerve agents proved relatively easy to resolve. The information bases are fairly complete, and there appears to be little risk either of adverse health effects from long-term exposure to low doses or of delayed health effects from acute exposures.'

Because there are gaps of knowledge regarding the potential health consequences of exposure to low (asymptomatic) concentrations of nerve agents, the Department of Veteran Affairs in cooperation with the Departments of Defense and Health and Human Services has initiated research projects in this area. These research projects are being conducted in laboratories almost exclusively outside the U.S. Government by both U.S. and international academic and industrial research organizations. It is hoped that a more complete picture of the health consequences of low-level OP nerve agent exposure will result from these efforts over the next 3 years.

References

- [1] Sidell F.R., Nerve Agents, in: Sidell F.R., Takafuji E.T., Franz D.R. (Eds.), Textbook of Military Medicine, Medical Aspects of Chemical and Biological Warfare, Office of the Surgeon General, US Army, 1997, p. 131.
- [2] Somani S.M., (Ed.), Chemical Warfare Agents, San Diego, Academic Press, 1992, pp. 156-194.
- [3] Medical Management of Chemical Casualties Handbook, Chemical Casualty Care Office, Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, 1995, p. 17.
- [4] Panel on Anticholinesterase Chemicals, Possible Long-Term Health Effects of Short-Term Exposure to Chemical Agents, Vol. I, Anticholinesterases and Anticholinergics, Committee on Toxicology and Environmental Health Hazards, Assembly of Life Sciences, Washington: National Academy Press, 1982.
- [5] Coordinating Subcommittee, Possible Long-Term Health Effects of Short-Term Exposure to Chemical Agents, Vol III, Final Report, Current Health Status of Test Subjects, Committee on Toxicology, Board on Toxicology and Environmental Health Hazards, Commission on Life Sciences, National Research Council, Washington National Academy Press, 1985.
- [6] Metcalf D.R., Holmes J.W., EEG, Psychological and neurological alterations in humans with organophosphorus exposure, *Ann. N.Y. Acad. Sci.* 160 (1969) 357-365.
- [7] Duffy F.H., Burchfiel J.L., Bartels P.H., Gaon M., Sim V.M., Long-term effects of an organophosphate upon the human electroencephalogram, *Toxicol. Appl. Pharmacol.* 47 (1979) 161-176.
- [8] Korsak R.J., Sato M.N., Effect of chronic organophosphate exposure on the central nervous system, *Clin. Toxicol.* 11 (1977) 83-85.
- [9] Burchfiel J.L., Duffy F.H., Sim V.M., Persistent effects of sarin and dieldrin upon the primate electroencephalogram, *Toxicol. Appl. Pharmacol.* 35 (1976) 365-379.
- [10] Shih T.A. et al., Metabolite pharmacokinetics of soman, sarin and GF in rats and biological monitoring of exposure to toxic organophosphorus agents, *J. Appl. Toxicol.* 14 (1994) 195-199.
- [11] Moore D.H., Low Dose Exposure and Long Term Health Effects of Nerve Agent at CBMTS Middle East I Symposium, December 7-11, Cairo, Egypt, 1997.
- [12] Kant G.J. et al., Effects of soman on neuroendocrine and immune function, *Neurotoxicol. Teratol.* 13 (1991) 223-228.
- [13] Goldman M. et al., Toxicity Studies on Agents GB and GD, University of California, Davis Laboratory for Energy-Related Health Research Final Report, AD-A187841, 1987.
- [14] LaBorde J.B. et al., Developmental Toxicity Study of Agent GB DCSM Types I and II in NZW Rabbits and CD Rats, Jefferson, AR, National Center for Toxicological Research Final Report, AD-A168331, 1986.
- [15] Bucci T.J. et al., Toxicity Studies on Agents GB and GD (Phase II): Delayed Neuropathy Study of Sarin, Type II, in SPF White Leghorn Chickens, Jefferson, AR, National Center for Toxicological Research Final Report, AD-A257183, 1992.
- [16] Sidell F.R. Hurst C.G., The Long-Term Health Effects of Nerve Agents and Mustard, in: Sidell F.R., Takafuji E.T., Franz D.R. (Eds.), Textbook of Military Medicine, Medical Aspects of Chemical and Biological Warfare, Office of the Surgeon General, US Army, 1997, pp. 232.

Cholinergic excitation induces activity-dependent electrophysiological and transcriptional responses in hippocampal slices

Alon Friedman^a, Daniela Kaufer^b, Lev Pavlovsky^a, Hermona Soreq^b

^a*Departments of Physiology and Neurosurgery, Faculty of Health Sciences, and Zlotowski Center for Neuroscience, Ben Gurion University, Beersheva, 84105 Israel*

^b*Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, 91904 Israel*

Abstract — To explore the correlations between short-term neurophysiological events initiated by over-activation of acetylcholine receptors, and long-lasting changes in brain function, we combined electrophysiology and PCR-based measurements in hippocampal slices or live mice subjected to stress or drug-induced cholinergic activation. Our findings reveal a common cascade of neuronal events resulting in delayed suppression of cholinergic transmission. (©Elsevier, Paris)

Résumé — **Réponses électrophysiologiques et transcriptionnelles induites par l'activation cholinergique dans l'hippocampe.** Pour explorer les corrélations entre des événements électrophysiologiques à court terme, initiés par une stimulation des récepteurs cholinergiques, et des modifications à long terme des fonctions cérébrales, nous avons utilisé des mesures électrophysiologiques et des mesures d'expression de gènes basées sur la PCR. Les modèles étaient des tranches perfusées d'hippocampe ou des souris vivantes exposées à des drogues. Nos résultats indiquent que le stress psychologique, tout comme les drogues anticholinergiques, produisent une cascade d'événements identiques, entraînant la suppression différée de la transmission cholinergique. (©Elsevier, Paris)

acetylcholinesterase inhibitors / hippocampal slices / molecular neurophysiology / muscarinic responses

1. Introduction

The importance of acetylcholine (ACh) in higher mammalian cognitive functions and behavioral responses is well documented [4, 8, 9]. Brief abnormalities in cholinergic transmission are sometimes associated with long-term changes in memory, mood and behavior; prolonged changes are notably associated with several neurologic diseases (e.g., Alzheimer's dementia). However, the molecular mechanisms which lead from transiently disturbed cholinergic transmission to long-lasting alterations in brain functions are not well understood. For example, acute psychological stress entails immediate activation of central nervous system (CNS) cholinergic pathways which results in excessive ACh release [16]. Likewise, experimental, therapeutic or occupational exposure to inhibitors of the ACh hydrolyzing enzyme, acetylcholinesterase (AChE), induces instantaneous elevation of ACh levels due to suppressed ACh hydrolysis. Thus, both these conditions elicit short-term hyper-activation of cholinergic receptors and also cause delayed and persistent ab-

normalities in CNS structure and function [3, 26]. In line with these earlier reports, we have recently observed both rapid and long-lasting changes in the expression of key cholinergic proteins in the cortex and hippocampus of mice, following either acute stress or exposure to cholinesterase inhibitors [17]. The transcriptional responses which initiated these changes were prevented by Na⁺ channel blockers as well as by intracellular Ca²⁺ chelators, suggesting that they were preceded by modulation in neuronal electrical activity. Receptor-ligand interactions may therefore activate signal transduction pathways, modulating the extent and duration of the cascade of events which connect hyperactivation to the long-term changes.

To explore this hypothesis, we studied the dose and time-dependent electrophysiological responses to various AChE inhibitors *in vitro* and investigated the potential role of these events in modifying CNS gene expression. To this end, we subjected hippocampal slices to controlled levels of anticholinesterases and characterized the resultant neurophysiological responses as well as the corresponding changes in mRNA transcripts of key cholinergic genes. Our findings reveal electrophysiological and molecular neuronal responses which are mediated by muscarinic receptors. These rapid, yet long-lasting changes may lead to permanent neurophysiological impairments.

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; CCh, carbamylcholine; CNS, central nervous system; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR.

2. Materials and methods

2.1. Brain slices

Mice were anesthetized and killed by decapitation, with brains quickly removed into ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl, 124; KCl, 3; MgSO₄, 2; NaH₂PO₄, 1.25; NaHCO₃, 26; D-glucose, 10; and CaCl₂, 2 (pH 7.4), saturated with 95% O₂, 5% CO₂. Corticohippocampal slices (400 µm thick) were cut in the sagittal plane using a vibratome (Vibroslice, Campden Instruments, Loughborough, UK), and were placed in a humidified holding chamber, continuously perfused with oxygenated aCSF at 37 °C. Drugs were added to the aCSF at the concentrations noted in the text.

2.2. Forced swim stress

Forced swim stress protocol was adapted for use in adult FVB/N mice as described [13]. Briefly, naive animals were subjected to two 4-min swim sessions separated by a 4-min interval in a 60 × 60 cm water bath (12 cm deep) at 21 ± 1 °C.

2.3. Electrophysiological recordings

Electrophysiological recordings were made using standard techniques [11, 12]. Extracellular recording electrodes were filled with a solution containing (in mM): NaCl, 140; KCl, 3; MgCl₂, 2; D-glucose, 10; CaCl₂, 2, and HEPES, 10 (pH 7.4). Electrodes (tip diameter 4–6 µm) were placed in the CA1 cell-body layer. Synaptic responses were evoked by de-

livering 20 µs stimuli through bipolar tungsten electrodes. Electrophysiological data were digitized on-line using Axotape (Axon Instruments, Inc.).

2.4. RNA extraction

Cerebral cortex and hippocampus from mouse brains were quickly dissected and placed in liquid nitrogen. Tissue was homogenized using a Thomas glass homogenizer and RNA extracted by the guanidinium thiocyanate RNA-Clean method (AGS, Heidelberg, Germany). For kinetic follow up of RT-PCR, PCR products were sampled every third cycle as detailed elsewhere [17]. Densitometric analysis was performed on ethidium-bromide stained agarose gels using the Adobe Photoshop software.

2.5. AChE activity measurements

Acetylthiocholine (ATCh) hydrolysis levels in brain homogenates were determined spectrophotometrically in triplicate and were expressed as nanomol ATCh per min per mg protein. The rate of values representing spontaneous ATCh hydrolysis was determined and subtracted.

3. Results

The time course and drug dependence of transcriptional responses associated with cholinergic activation were detected by reverse-transcriptase PCR

Table I. RT-PCR density from stressed mice and slices exposed to anti-AChEs^a.

Gene	Treatment	Post insult time (min)	mRNA level (% of control)	PCR cycle of first appearance (mean)	n	P (t-test) (compared to control)
c-Fos	Control	10	100 ± 33	28 ± 1.6	7	
	Anti-ChEs	10	380 ± 275	25 ± 1.7	4	0.003
	Stress	10	401 ± 151	25 ± 1.5	5	0.011
ChAT	Control	30	100 ± 43	28 ± 2.0	15	
	Anti-ChEs	30	30 ± 1933	31 ± 1.6	5	0.019
	Stress	30	19 ± 9	32 ± 1.3	6	0.027
VACHT	Control	30	100 ± 37	30 ± 0.0	2	
	Anti-ChEs	30	46 ± 0	33 ± 0.0	2	
	Stress	30	37 ± 8	33 ± 0.0	2	
AChE	Control	30	100 ± 4	31 ± 1.7	3	
	Anti-ChEs	30	877 ± 97	22 ± 1.7	2	0.001
	Stress	30	275 ± 93	24 ± 0.0	5	0.029
Synaptophysin	Control	30	100 ± 5	25 ± 1.5	13	
	Anti-ChEs	30	87 ± 8	25 ± 1.6	5	n.s.
	Stress	30	87 ± 15	25 ± 1.6	6	n.s.

^aRT-PCR was performed on RNA extracted from cortico-hippocampal slices incubated with 1 µM DFP or 1 mM pyridostigmine or from cortices of control or stressed mice. Control cortices were indistinguishable from mouse slice values. Products were sampled every third PCR cycle, electrophoresed, and stained with ethidium bromide. Presented are the relative band intensities as percent of control levels ± S.D. Values were calculated from three densitometric analyses of first visible PCR cycle of appearance. *n* equals the number of animals in each experimental group. *P* values were calculated using Student's *t*-test, for each treatment compared to its own control group.

kinetics (RT-PCR). Earlier cycle of product first appearance in this test reflects higher levels of the corresponding mRNA; within the linear phase of product accumulation, each cycle represents a two-fold increase in mRNA amounts [17]. Table 1 summarizes these RT-PCR analyses as referring to the mRNA level of several key genes under experimental conditions (stress in vivo or anticholinesterase exposure in slices) compared to control in vivo or slice conditions. In agreement with the definition of c-fos as an early immediate gene [14], we observed four-fold increases in c-fos mRNA levels as soon as 10 min post insult, the insult being either stress or exposure to an anti-AChE. This increase potentially elevates the amount of the resultant c-fos transcription factor, and thus changes the transcriptional efficacy of promoters. The choline acetyl transferase promoter, which directs expression of the ACh synthesizing enzyme, ChAT [1], and the co-regulated vesicular acetylcholine transporter, VAcHT [5] and the AChE promoter [2, 19] both belong to this class of c-fos responders. This suggested that ACh availability might be subject to c-fos regulation, but we could not predict in what direction, since c-fos may either enhance or suppress gene expression depending on its modular interactions with various Jun proteins.

RT-PCR analyses demonstrated 2–3- and 3–5-fold reductions for ChAT and VAcHT mRNAs under anticholinesterase exposure or stress, respectively. In contrast, AChE mRNA revealed an opposite trend of nine- and three-fold increases for anti-cholinesterase and stress exposure, respectively. Similar analyses demonstrated no significant change for synaptophysin mRNA, indicating a selectivity of the observed modulations for cholinergic elements (table 1). Intraperitoneal injection of a variety of AChE inhibitors induced changes in brain mRNA levels which were parallel to those observed in brain slices (data not shown). These findings suggested that the c-fos mediated transcriptional responses reflected a consensus consequence to the CNS increase in ACh levels following blockade of AChE's catalytic activity and demonstrated the validity of the in vitro brain slices system for parallel electrophysiological and molecular analyses of the direct effects of AChE inhibitors (see Kaufer et al. [18] for further details of this model system).

To explore the physiological consequences associated with the above transcriptional modulations, we recorded evoked potentials in the pyramidal cell layer of the hippocampal CA1 region. Brief stimulus to the stratum oriens, containing septo-hippocampal afferent cholinergic fibers [6, 25, 27], under normal conditions evoked a typical synaptic potential and population spike; amplitudes of both were proportional to stimulus intensity. Exposure of slices to py-

ridostigmine resulted in a dose-dependent increase in population spike amplitude (figure 1A,B). Increased spike amplitudes were measured over a wide range of stimulus intensities, without affecting the maximal amplitude (figure 1B). This increase in population spike amplitude plateaued at pyridostigmine doses above 10 μ M, which paralleled exactly the extent of AChE inhibition by pyridostigmine in cortex homogenates: catalytic activity measurements over the same ranges of pyridostigmine concentrations revealed inhibition of 25% to > 95% of AChE activity, with plateau over 10 μ M (figure 1B, inset and Friedman et al. [13]). These findings support the notion that the electrophysiological effects of pyridostigmine were associated with decreased AChE catalytic activity. That the observed response to this AChE inhibitor was indeed due to increased concentrations of unhydrolyzed, released ACh, was further supported by the dose-dependent increases in population spike amplitude with increasing concentrations of the cholinergic agonist carbamylcholine (CCh) (figure 1C). Similar responses were observed using other AChE-inhibitors, including physostigmine (10 μ M) (figure 1D) or diisopropyl fluorophosphate (DFP) (1 μ M) ([18] and data not shown). In all cases, the augmented response was reversed when a muscarinic antagonist (atropine, 5 μ M) was added to the aCSF (figure 1D).

Acetylcholine is known to modify membrane properties of neurons via slow second-messenger dependent processes [7, 21, 23]. Thus, prevention of ACh hydrolysis in the synaptic cleft is expected to alter incoming stimuli in a frequency-dependent manner. Therefore, we tested the effects of anti-AChEs on responses to repeated stimulation. Under low stimulus intensities, evoked synaptic responses did not evoke population spikes either in normal solution or following pyridostigmine (10^{-4} M) application (figure 2A). However, under these low stimulation levels, pyridostigmine application resulted in a prominent facilitation of the second stimulus at inter-stimulus intervals of 30–200 ms (figure 2A). The expected duration of up to 200 ms for these paired-pulse facilitation responses under normal conditions characterizes afferent excitatory connections to the CA1 region [10, 20]. High doses of AChE inhibitors extended this duration to over 500 ms (data not shown and see Kaufer et al. [18]). With 10 repetitive stimuli at 40 Hz, physostigmine further induced a large and prolonged negative deflection in the extracellular recording (figure 2B), indication of influx of positive ions into nearby neurons.

The transcriptional responses following AChE inhibition preceded an increase in AChE activity (see [17]). Therefore, we expected that anticholinesterase-induced increased excitability will be followed

by a delayed phase of suppressed neuronal excitability. *Figure 2C* shows that exposure to physostigmine prompted, within 1 hr, increased population spike amplitudes. When we extended AChE inhibition to 3 h, the augmented synaptic response as well as action potentials were significantly muted. These observations demonstrated that AChE inhibitors mediate a transient, early phase of enhanced excitability that is followed by a secondary phase of suppressed neuronal activity. Both phases were prevented by the presence of atropine (*figure 1D* and see Kaufer et al. [17]), confirming their dependence on muscarinic receptors activation.

4. Discussion and conclusion

Using hippocampal mouse brain slices, we performed an in-depth analysis of the short-term changes in the evoked electrophysiological events following exposure to various anti-cholinesterases or ACh analogues. In parallel, we examined in these slices the levels of mRNA transcripts encoding key cholinergic elements, and compared the changes in these transcript levels to those occurring *in vivo* under acute exposure to anti-AChEs or psychological stress. Together, these experiments suggest a convergent pathway leading from immediate elevation in

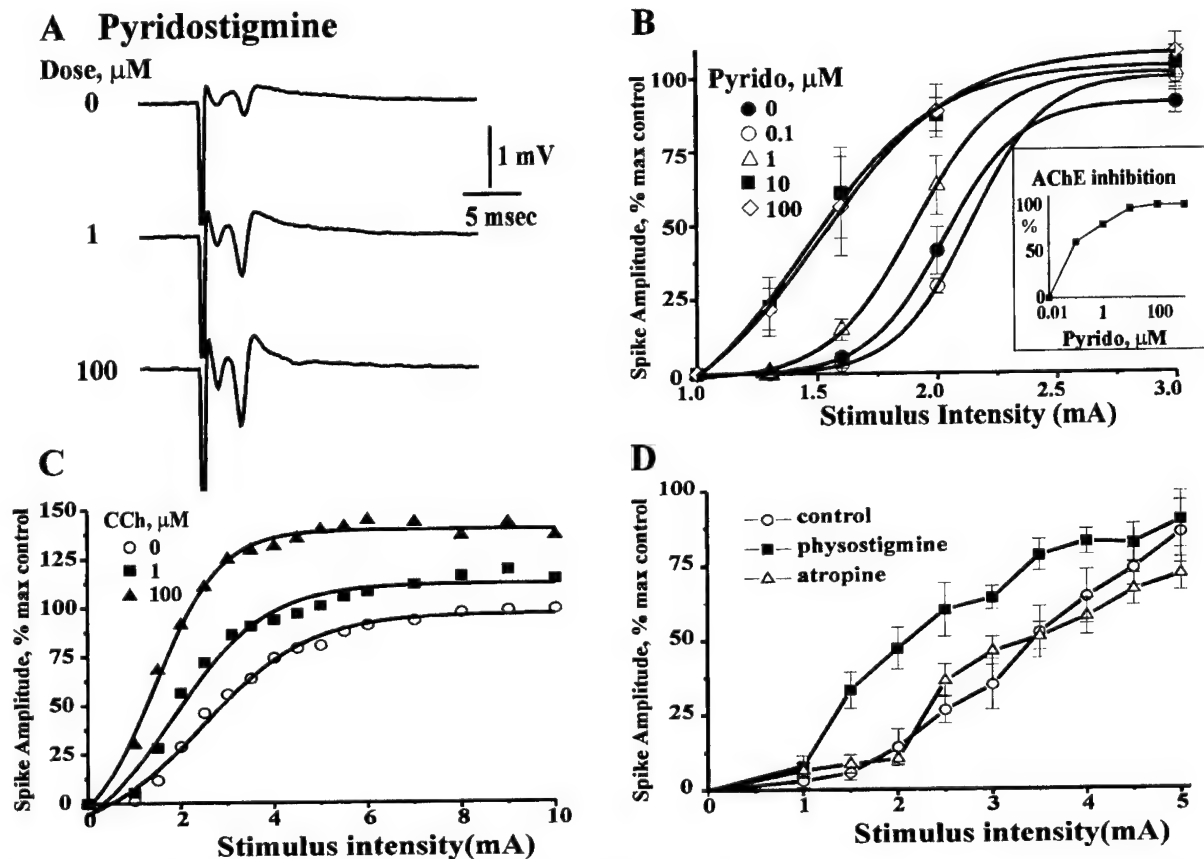


Figure 1. Muscarinic-dependent increases in neuronal excitability in sagittal hippocampal slices maintained *in vitro*. **A.** Dose-dependent increases in evoked population spikes. Recordings represent activities in hippocampal slices exposed to the noted doses of pyridostigmine for one hour. **B.** Spike amplitude increases under pyridostigmine. Spike amplitudes were measured under the noted stimulus intensities for pyridostigmine concentrations over the range of 0.1–100 μM . Inset, measurements of AChE activities in homogenates reveal inhibition in the same concentration range of pyridostigmine. **C.** Response to ACh analogue. Dose-dependent increases in population spike amplitudes are presented for slices exposed to the noted concentrations of the cholinergic agonist carbamylcholine (CCh). **D.** Muscarinic-dependence of anti-AChE effect. The increase in evoked response observed following 10 μM physostigmine (filled squares), as compared to control conditions (open circles) was reversible upon the administration of the muscarinic antagonist, atropine (open triangles).

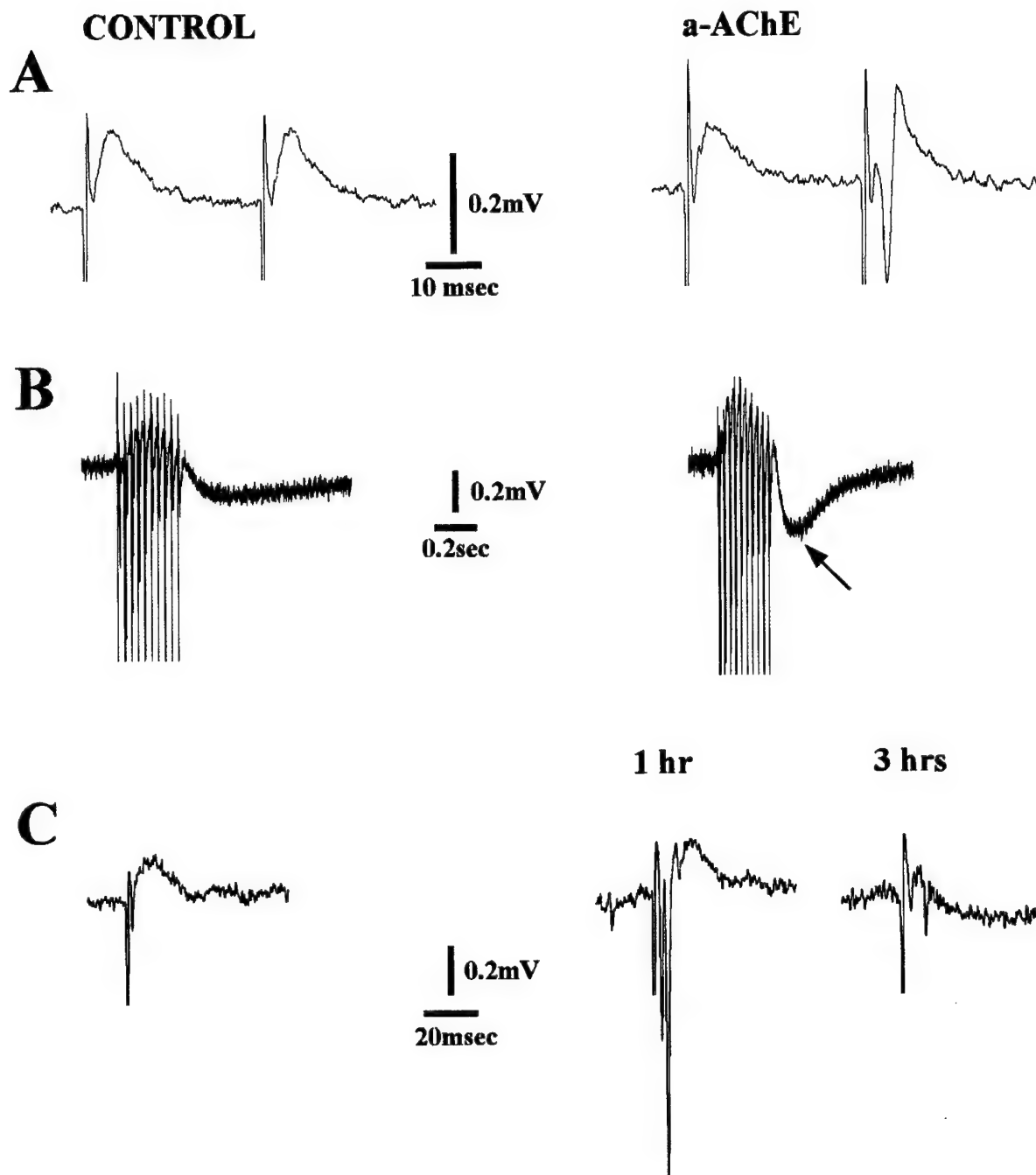


Figure 2. Effect of anti-cholinesterases is time- and stimulus-dependent. **A.** Paired pulse responses to low-intensity stimuli. Normal responses were recorded at 30 ms interval, 0.3 mA, under control conditions or following 1 h perfusion with pyridostigmine. Note the lack of facilitation of the synaptic currents under control conditions, and the prominent facilitation following pyridostigmine (a-AChE), observed as stimulus triggered population spike. Plotted traces are average of six consecutive stimuli. **B.** Modulated response to repetitive stimulation. Ten repetitive stimuli of the stratum-oriens at 40 Hz evoked responses which were first facilitated, then depressed and followed by a slow negative potential. Following 1 h of exposure to 10 μ M physostigmine (a-AChE), population spike and facilitation increased, depression decreased, and the following slow current was prominently enhanced in amplitude and duration (arrow). **C.** Time dependent changes in population spike amplitudes. Increases were observed 1 h following exposure but not at 3 h (see text).

ACh levels to delayed suppression of cholinergic neurotransmission.

Analysis of our electrophysiological measurements reveals several intriguing points. First, we noted that both the increase in population spike amplitudes and the inhibition of AChE's catalytic activity reached plateau levels at similar pyridostigmine concentrations (around 10^{-5} M). This strongly supports the notion that the inhibition of AChE-mediated ACh hydrolysis was the cause of these physiological responses. Moreover, a non-hydrolyzable ACh analogue caused similar augmentation in excitability, and the augmented synaptic responses were reversible upon atropine administration. Therefore, we conclude that released ACh enhances CA1 output.

It is noteworthy that a difference between the effect of cholinesterase inhibitors (e.g., pyridostigmine or physostigmine) and that of the ACh analogue, CCh, on population spike amplitudes was observed. Anticholinesterases increased spike amplitudes without exceeding the maximal amplitude under control conditions, whereas CCh elevated spike amplitudes in a dose-dependent manner to levels above those measured for controls. This suggests that perfused CCh, but not stimulus-dependent released ACh, recruits more neurons to fire action potentials in response to the maximal employed stimulus. Cutting the long septo-hippocampal afferents in the preparation of our slices may have decreased cholinergic input on CA1 neurons and be the basis for this observed difference. In support of this, preliminary experiments showed hippocampal slices cut on the coronal plane to respond poorly to anti-AChEs, compared to sagittal slices, with no difference between preparations in their response to CCh.

Different mechanisms have been proposed for the action of ACh on CA1 pyramidal neurons. The observed enhancement of stimulus evoked response could be the result of the following cholinergic-dependent mechanisms: 1) increased glutamate release [15]; 2) increased post-synaptic response to the released glutamate [22]; 3) modulation of local synaptic inhibition [24]; and/or 4) decreased threshold of post-synaptic action potentials. This last option is particularly attractive, since under low-intensity stimuli, released ACh did not significantly alter the synaptic current. Moreover, the stimulus-dependent enhancement of evoked responses following exposure to anti-AChEs probably reflects the slow, second messenger-dependent actions of ACh on membrane conductances via activation of muscarinic receptors (see for example [7, 21]).

Reduced firing threshold following anti-AChE exposure is often associated with additional spikes as

well as with late inward currents following repetitive stimulation (*figure 2B*). This indicates prolonged depolarization, which is expected to be associated with increased Ca^{2+} influx through NMDA- and voltage-dependent channels. Pharmacological manipulation demonstrated that changes in transcription following anti-AChEs are preventable by the intracellular Ca^{2+} chelator, BAPTA-AM. This points to the importance of Ca^{2+} as an intracellular second messenger, bridging rapid modulations in electrical activity to long-term changes in the structure and function of the cholinergic synapse.

Acknowledgments

This study has been supported by the Teva Research prize for young investigators, the Smith Psychobiology Fund and the Goldman Fund (to A.F.) as well as the by the Basic Research Fund (to H.S.). All experiments were performed in accordance with institutional guidelines for animal experimentation.

References

- [1] Bausero P., Schmitt M., Toussaint J.L., Simoni P., Geoffroy V., Queuche D., Duclaud S., Kempf J., Quirin-Stricker C., Identification and analysis of the human choline acetyltransferase gene promoter, *Neuroreport* 4 (1993) 287–290.
- [2] Ben Aziz-Aloya R., Seidman S., Timberg R., Sternfeld M., Zakut H., Soreq H., Expression of a human acetylcholinesterase promoter-reporter construct in developing neuromuscular junctions of *Xenopus* embryos, *Proc. Natl. Acad. Sci. USA* 90 (1993) 2471–2475.
- [3] Bremner J.D., Randall P., Scott T.M., Bronen R.A., Seibyl J.P., Southwick S.M., Delaney R.C., McCarthy G., Charney D.S., Innis R.B., MRI-based measurement of hippocampal volume in patients with combat-related posttraumatic stress disorder, *Am. J. Psychiatry* 152 (1995) 973–981.
- [4] Buresova O., Bures J., Bohddanecky Z., Weiss T., The effect of atropine on learning, extinction, retention and retrieval in rats, *Psychopharmacologia* 5 (1964) 255–263.
- [5] Cervini R., Houhou L., Pradat P.F., Bejanin S., Mallet J., Berrard S., Specific vesicular acetylcholine transporter promoters lie within the first intron of the rat choline acetyltransferase gene, *J. Biol. Chem.* 270 (1995) 24654–24657.
- [6] Cole A.E., Nicoll R.A., Acetylcholine mediates a slow synaptic potential in hippocampal pyramidal cells, *Science* 221 (1983) 1299–1301.
- [7] Cole A.E., Nicoll R.A., Characterization of a slow cholinergic post-synaptic potential recorded in vitro from rat hippocampal pyramidal cells, *J. Physiol. (Lond.)* 352 (1984) 173–88.
- [8] Deutch J.A., The cholinergic synapse and the site of memory, *Science* 174 (1971) 788–794.
- [9] Drachman D.A., Leavitt J., Human memory and the cholinergic system, *Arch. Neurol. Chicago* 30 (1974) 113–121.
- [10] Dunwiddie T.V., Haas H.L., Adenosine increases synaptic facilitation in the in-vitro rat hippocampus: evidence for a presynaptic site of action, *J. Physiol. (Lond.)* 369 (1985) 365–377.

- [11] Fleidervish I., Friedman A., Gutnick M.J., Slow Na channel inactivation underlies cumulative slow adaptation of neuronal firing in guinea-pig and mouse neocortical slices in vitro, *J. Physiol. (Lond.)* 493 (1996) 83–98.
- [12] Friedman A., Gutnick M.J., Intracellular calcium and control of burst generation in neurons of guinea-pig neocortex in vitro, *Eur. J. Neurosci.* 1 (1989) 374–381.
- [13] Friedman A., Kaufer D., Shemer J., Hendler L., Soreq H., Tur-Kaspa I., Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response, *Nature Med.* 2 (1996) 1382–1385.
- [14] Ghosh A., Ginty D.D., Bading H., Greenberg M.E., Calcium regulation of gene expression in neuronal cells, *J. Neurobiol.* 25 (1994) 294–303.
- [15] Gray R., Rajan A.S., Radcliffe K.A., Yakehiro M., Dani J.A., Hippocampal synaptic transmission enhanced by low concentrations of nicotine, *Nature* 383 (1996) 713–716.
- [16] Imperato A., Puglisi-Allegra S., Casolini P., Angellucci L., Changes in brain dopamine and acetylcholine release during and following stress are independent of the pituitary-adrenocortical axis, *Brain Res.* 538 (1991) 111–117.
- [17] Kaufer D., Friedman A., Seidman S., Soreq H., Acute stress facilitates long-lasting changes in cholinergic gene expression, *Nature* 393 (1998) 373–377.
- [18] Kaufer D., Friedman A., Seidman S., Soreq H., Anticholinesterases induce multigenic transcriptional feedback response suppressing cholinergic neurotransmission, *Chem. Biol. Int.* (1998), in press.
- [19] Li Y., Camp S., Rachinsky T.L., Bongiorno C., Taylor P., Promoter elements and transcriptional control of the mouse acetylcholinesterase gene, *J. Biol. Chem.* 268 (1993) 3563–3572.
- [20] Leung L.S., Fu X.W., Factors affecting paired-pulse facilitation in hippocampal CA1 neurons in vitro, *Brain Res.* 650 (1994) 75–84.
- [21] Malenka R.C., Madison D.V., Andrade R., Nicoll R.A., Phorbol esters mimic some cholinergic actions in hippocampal pyramidal neurons, *J. Neurosci.* 6 (1986) 475–480.
- [22] Markram H., Segal M., The inositol 1,4,5-trisphosphate pathway mediates cholinergic potentiation of rat hippocampal neuronal responses to NMDA, *J. Physiol. (Lond.)* 447 (1992) 513–533.
- [23] Nicoll R.A., The coupling of neurotransmitter receptors to ion channels in the brain, *Science* 241 (1988) 545–551.
- [24] Pitler T.A., Alger B.E., Cholinergic excitation of GABAergic interneurons in the rat hippocampal slice, *J. Physiol. (Lond.)* 450 (1992) 127–142.
- [25] Schafer M.K.H., Eiden L.E., Weihe E., Cholinergic neurons and terminal fields revealed by immunohistochemistry for the vesicular acetylcholine transporter, *Neuroscience* 84 (1998) 331–359.
- [26] Sapolsky R.M., Why stress is bad for your brain?, *Science* 273 (1996) 749–750.
- [27] Wainer H., Mesulam M.M., Ascending cholinergic pathways in the rat brain, in: Striade M., Biesold D. (Eds.), *Brain Cholinergic Systems*, Oxford Science Publications, 1990, pp. 65–130.

Novel m1 muscarinic agonists in treatment and delaying the progression of Alzheimer's disease: An unifying hypothesis

Abraham Fisher, Rachel Brandeis, Rachel Haring, Naomi Eshhar, Eliahu Heldman, Yishai Karton, Orli Eisenberg, Haim Meshulam, Daniele Marciano, Nira Bar-Ner, Zipora Pittel

Israel Institute for Biological Research, PO Box 19, Ness-Ziona 74100, Israel

Abstract — M1 selective agonists from the AF series (e.g. AF102B, AF150(S)), via m1 muscarinic receptors, activate distinct signal transductions, enhance amyloid precursor proteins secretion from transfected cells and primary cell cultures, show neurotrophic effects and are beneficial in a variety of animal models for Alzheimer's disease. Such m1 agonists may be effective in the treatment and therapy of Alzheimer's disease. (©Elsevier, Paris)

Résumé — Les agonistes sélectifs de type M1 appartenant à la série AF (par exemple AF102B, AF150(S)) activent de manière sélective certaines routes de transduction du signal via les récepteurs muscariniques de type m1, augmentent la sécrétion de la protéine amyloïde par les cellules transfectées et les cultures primaires de cellules, produisent des effets neurotrophiques et se montrent bénéfiques pour une variété de modèles animaux de la maladie d'Alzheimer. De tels agonistes peuvent être efficaces pour traiter et guérir la maladie d'Alzheimer. (©Elsevier, Paris).

AF series / m1 agonists / Alzheimer's disease

1. Introduction

An m1 selective muscarinic agonist may be effective in treatment of Alzheimer's disease (AD), regardless of the extent of degeneration of presynaptic cholinergic projections to the frontal cortex and hippocampus (for review see [4]).

Mismetabolism of amyloid precursor proteins (APP) may induce AD via increased formation of β -amyloids (A β). However, activation of the non-amyloidogenic pathway may be beneficial in the treatment of AD, since upon activation of this pathway A β formation is reduced [3, 17]. This non-amyloidogenic pathway can be enhanced via activation of m1 mAChR [3, 9, 10, 17, 18]. The cholinergic hypofunction in AD can be tied with formation of A β and these can impair the coupling of m1 muscarinic receptors (m1 mAChR) with G-proteins [15]. This uncoupling leads to decreased signal transductions, impairments in cognition, a reduction in levels of trophic secreted APP, generation of more neurotoxic β -amyloids and a further decrease in acetylcholine release. This 'vicious cycle' may be prevented in principle by m1 selective agonists. Such m1 agonists are found in the AF series (e.g., project drugs: AF102B, AF150(S)) [4, 5]. The following text is an overview of recent findings with these compounds.

2. Select signal transduction by the AF series

The AF series are functionally selective m1 agonists (e.g., on $[Ca^{2+}]_i$ elevation, phosphoinositides hydrolysis or arachidonic acid release), yet antagonists in elevating cAMP levels in cell cultures transfected with m1 mAChR [5]. These m1 agonists may activate distinct sets of G-proteins and extend drug selectivity beyond the ligand recognition site. This property might be beneficial clinically due to altered signal transductions in AD. In this context, both mRNA for Gs α (which mediates activation of adenylyl cyclase and enhanced release of cAMP) and m1 mAChR were reported to be elevated in hippocampi of AD patients [13, 14]. We propose that AD treatment by m1 agonists should not stimulate adenylyl cyclase via m1 mAChR but should activate PI hydrolysis [5, 7]. In the event that this hypothesis is valid, concerns over the long-term use of highly efficacious muscarinic agonists, which activate all m1 mAChR-mediated signal transductions, including m1 mAChR coupling with Gs, leading eventually to an increased cAMP level in the brain, will be raised. The same caution should be applied to acetylcholinesterase (AChE) inhibitors for the long-term treatment of AD since in this case elevated acetylcholine levels, due to AChE inhibition, can again enhance brain cAMP levels.

3. Processing of APP and elevation of secreted APPs

We used PC12M1 cells, which express both kinase tyrosine receptors and m1 mAChR in order to investigate the effects of m1 agonists on levels of secreted APPs and the role of various neurotrophins on m1 mAChR-mediated effects. We found that m1 selective muscarinic agonists, such as AF102B [9] and AF150(S) (unpublished results) increase APPs secretion via the non-amyloidogenic pathway, without desensitizing the m1 mAChR. In addition, nerve growth factor (NGF) augments muscarinic-induced APPs release and reduces membrane-associated APP [10]. This activation is also augmented by other neurotrophins such as basic fibroblast growth factor (bFGF). Some signal-transduction pathways activated by muscarinic agonists and by growth factors, respectively, may cross-react, resulting in an augmentation of the response at their convergence point. The muscarinic-induced APPs secretion is mediated by several transduction pathways which operate in parallel and involve PKC-dependent and *ras*-dependent cascades. Additional stimulation of m1 mAChR by muscarinic agonists in these cells results in activation of mitogen activated protein kinases (MAPK) which is *ras*-dependent and PKC-independent and is synergistically enhanced by neurotrophins [11, 12].

4. Effect of cholinergic stimulation on APPs level in embryonic rat brain – primary cell cultures

Primary cell cultures of hippocampus and cerebral cortex derived from 18–19 days old fetuses were used in this study. The effect of 100 μ M of carbachol, a non-selective muscarinic agonist and two m1 functionally selective muscarinic agonists AF102B and AF150(S) on APPs secretion was evaluated. Blockade of their effects were tested in presence of 10 μ M pirenzepine, an m1 selective antagonist or 50 μ M gallamine, an m2 antagonist. We found that these muscarinic agonists significantly increased (2–6-fold over control) APPs secretion after 1 h of incubation (figure 1). These effects were completely blocked by pirenzepine, indicating that activation of m1 mAChR subtype may be responsible for the elevated APPs secretion. Notably, these data corroborate our results from rat cortical brain slices [18].

5. Neurotrophic and neuroprotective effects

The AF series induce neurotrophic-like effects, which are synergistic with a variety of neurotrophins (NGF [8]), bFGF, epidermal growth factor [11]) and can promote survival of cultured primary CNS

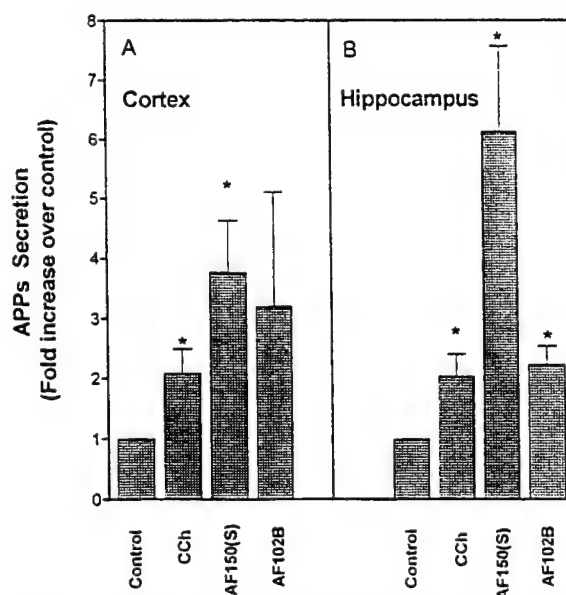


Figure 1. APPs secretion from rat cortical (A) and hippocampal (B) primary cell cultures by 100 μ M carbachol (CCh), AF102B and AF150(S). Data were normalized according to control values which were determined in absence of muscarinic agonists. Data represent 2–6 individual experiments and are presented as means \pm SEM. * P < 0.05 relative to control evaluated by paired Student *t*-test.

neurons [1]. Like in case of the non-amyloidogenic APP processing pathways, these m1 agonists promote and exert neurotrophic activities in conjunction with some neurotrophine-dependent and -independent signal(s) [11].

6. Effects of the AF series in animal models for AD

AF102B and AF150(S) restored memory and learning deficits in a variety of animal models that mimic cholinergic deficits in AD, without producing adverse central and peripheral side-effects at effective doses and showing a relatively wide-safety margin (for AF150(S) see table I; for other compounds from this series see [4, 5]). Notably, AF150(S) restored working memory impairments to normal in apolipoprotein E-deficient mice [6]. Furthermore, this cognitive improvement was associated with a parallel recovery of brain cholinergic markers (choline acetyltransferase (ChAT), AChE and m1 mAChR) [6], as well as a reduction of tau protein hyperphosphorylation [16]. Thus, AF150(S) emerges as a promising candidate drug for the treatment of AD.

Table I. AF150(S), a compilation of pharmacodynamic and behavioral profiles.

	<i>Dose (mg/kg, p.o.)</i>	<i>Effects (mAChR subtype mediated)</i>
<i>Peripheral nervous system effects [2]</i>		
Redness around mouth	50	(?)
Salivation	100	(M1, M3)
Chromodacryorrhea	200	(M3?)
Diarrhea	≥ 500	no effect (M3)
<i>Central nervous system effects</i>		
Sedation	200	(?)
Tremors	500	(M2, M4)
Hypothermia	500	(M2)
Open field	1–5	no effect
<i>Memory and learning</i>		
Social memory (naive rats)^a	1, 5	positive effect
AF64A-rats [2]		
Passive avoidance	0.3, 0.5, 1	positive effect
MWM-RM	0.5, 1, 5	positive effects
MWM-WM	0.2 (chronic)	tendency for positive effects
RAM	0.5, 5	no effects
	1	positive effects
Social memory ^a	1	positive effects
Ischemia-rats^a		
MWM (WM)	0.5 (chronic)	positive effects
Apolipoprotein E-deficient mice [6, 17]		
MWM (WM)	0.5 (chronic)	positive effects on MWM-WM, ChAT, AChE, m1 mAChR, overphosphorylation of <i>Tau</i>
Cholinergic markers		
<i>Tau</i> phosphorylation		
Pigeons [19]		
Scopolamine (0.03 mg/kg, i.p.) induced deficits in DMTS	4 (i.p.)	positive effects

^aBrandeis et al., unpublished results.

RAM, eight-armed radial maze; DMTS, delayed matching-to-sample; i.p., intraperitoneal; p.o., oral; MWM, Morris water maze; RM, reference memory; WM, working memory.

7. Conclusions

The m1 agonists from the AF series operate by several mechanisms acting in concert to positively affect cholinergic neurotransmission, neuronal survival and, ultimately, restore and preserve mnemonic processes in a variety of animal models which mimic the cholinergic hypofunction in AD. Apart from directly boosting the cholinergic signal, these novel compounds have the added advantage of positively acting, via m1 mAChR receptors, on other target systems which are central to the pathogenesis of several neurodegenerative diseases, including AD. For example, the three hallmarks of AD, cholinergic deficiency, A β and hyperphosphorylated *tau* proteins have been shown to be potential targets for these compounds (for review see [4]). AF150(S) and AF102B have the potential of reducing the formation of A β and preventing A β -induced neurotoxicity. Notably, AF150(S) and AF102B show neurotrophic effects and increase secretion of APPs which are both neuroprotective and neurotrophic. This may be im-

portant in compensating for possible dysfunctions of neurotrophins in AD. Due to their effects on *tau* dephosphorylation, such m1 agonists may impair paired helical filaments formation [16, 20]. Finally, due to the positive role of m1 mAChR on most of the identified 'villains' and risk factors in AD, including apoptosis (unpublished results), such m1 agonists may represent a unique therapeutic strategy in AD. Long term use of m1 agonists in early stage of AD patients and/or other populations at risk are needed to test this unifying hypothesis both in treatment and therapy of AD.

References

- [1] Albrecht J., Gurwitz D., Fisher A., Mount H.T.J., Novel muscarinic m1 receptor agonists promote survival of CNS neurons in primary cell culture, Soc. Neurosci. Abst. 21 (1995) 2040.
- [2] Brandeis R., Sapir M., Hafif N., Abraham S., Oz N., Stein E., Fisher A., AF150(S): A new functionally selective M1

- agonist improved cognitive performance in rats, *Pharmacol. Biochem. Behav.* 51 (1995) 667–674.
- [3] Buxbaum J.D., Oishi M., Chen H.I., Pinkas-Kramarski R., Jaffe E.A., Gandy S.E., Greengard P., Cholinergic agonists and interleukin regulate processing and secretion of the Alzheimer β A4 amyloid protein precursor, *Proc. Natl. Acad. Sci. USA* 89 (1992) 10075–10078.
 - [4] Fisher A., Muscarinic agonists for the treatment of Alzheimer's disease: progress and perspectives. Review, *Eur. Opin. Invest. Drugs* 6 (1997) 1395–1411.
 - [5] Fisher A., Barak D., Progress and perspectives in new muscarinic agonists, *Drug. News Perspect.* 7 (1994) 453–464.
 - [6] Fisher A., Brandeis R., Chapman S., Pittel Z., Michaelson D.M., M1 muscarinic agonist treatment reverses cognitive and cholinergic impairments of apolipoprotein E-deficient mice, *J. Neurochem.* 70 (1998) 1991–1997.
 - [7] Gurwitz D., Haring R., Heldman E., Fraser M.C., Manor C.M., Fisher A. Discrete activation of transduction pathways associated with acetylcholine m1 receptor by several muscarinic ligands, *Eur. J. Pharmacol. (Mol. Pharmacol.)* 267 (1994) 21–31.
 - [8] Gurwitz D., Haring R., Heldman E., Pinkas-Kramarski R., Stein R., Fisher A., NGF-dependent neurotrophic-like effects of AF102B, an m1 muscarinic agonist, in PC12M1 cells, *NeuroReport* 6 (1995) 485–488.
 - [9] Haring R., Gurwitz D., Barg J., Pinkas-Kramarski R., Heldman E., Pittel Z., Wengier A., Meshulam H., Marciano D., Karton Y., Fisher A. Amyloid precursor protein secretion via muscarinic receptors: reduced desensitization using the M1-selective agonist AF102B, *Biochem. Biophys. Res. Commun.* 203 (1994) 652–658.
 - [10] Haring R., Gurwitz D., Barg J., Pinkas-Kramarski R., Stein R., Heldman E., Pittel Z., Danenberg D.G.D., Wengier A., Meshulam H., Marciano D., Karton Y., Fisher A., NGF promotes amyloid precursor protein secretion via muscarinic receptor activation, *Biochem. Biophys. Res. Commun.* 213 (1995) 15–23.
 - [11] Haring R., Heldman E., Pittel Z., Kloog Y., Marciano D., Fisher A., Transduction pathways mediating muscarinic stimulated amyloid precursor protein secretion and cell differentiation, *J. Neurochem.* 66 (Suppl) 2 (1996) S12B.
 - [12] Haring R., Fisher A., Marciano D., Pittel Z., Kloog Y., Zuckerman A., Eshhar N., Heldman E., Mitogen-activated protein kinase-dependent and protein kinase-C dependent pathways link the m1 muscarinic receptor to β -amyloid precursor protein secretion, *J. Neurochem.* (1998), in press.
 - [13] Harrison P.J., Barton A.J.L., McDonald B., Pearson R.C.A., Alzheimer's disease: Specific increases in a G-protein subunit (G α) mRNA in hippocampal and cortical neurons, *Mol. Brain Res.* 10 (1991) 71–81.
 - [14] Harrison P.J., Barton A.J.L., Najlerahim A., McDonald B., Pearson R.C.A., Increased muscarinic receptor messenger RNA in Alzheimer's disease temporal cortex demonstrated by in situ hybridization histochemistry, *Brain Res.* 9 (1991) 515–521.
 - [15] Jope R.S., Cholinergic muscarinic receptor signalling by phosphoinositides signal transduction system in Alzheimer's disease, *Alzheimer's Dis. Rev.* 1 (1996) 2–14.
 - [16] Michaelson D.M., Fisher A., Pharmacological reversal of neurochemical and cognitive derangements in apolipoprotein E-deficient mice, Fifth International Geneva/Springfield Symposium on Advances in Alzheimer Therapy, Geneva, April 15–19, 1998.
 - [17] Nitsch R.M., Slack B.E., Wurtman R.J., Growdon J.H., Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors, *Science* 258 (1992) 304–307.
 - [18] Pittel Z., Heldman E., Barg J., Haring R., Fisher A., Muscarinic control of amyloid precursor protein secretion in rat cerebral cortex and cerebellum, *Brain Res.* 742 (1996) 299–304.
 - [19] Ruske A.C., Fisher A., White K.G., Attenuation of scopolamine-induced deficits in delayed-matching performance by a new muscarinic agonist, *Psychobiology* 25 (1997) 313–320.
 - [20] Sadot W., Gurwitz D., Barg D., Behar L., Gizbur I., Fisher A., Activation of m1 muscarinic acetylcholine receptor regulates tau phosphorylation in transfected PC12 cells, *J. Neurochem.* 66 (1996) 877–880.

Molecular interactions of acetylcholinesterase with senile plaques

Nibaldo C. Inestrosa, Rodrigo Alarcón

*Departamento de Biología Celular y Molecular, Facultad de Ciencias Biológicas,
Pontificia Universidad Católica de Chile, Alameda 340, P.O. Box 114-D, Santiago, Chile*

Abstract — Acetylcholinesterase (AChE) present in Alzheimer plaques is resistant to low pH, anti-ChE inhibitors and high substrate concentrations in comparison with the free enzyme. Kinetic and pharmacological studies of AChE-amyloid complexes indicate that steric hindrance by the amyloid over the gorge and the peripheral site of AChE is responsible for these effects. (©Elsevier, Paris)

Résumé — Interactions moléculaires de l'acétylcholinestérase avec les plaques séniles. L'acétylcholinestérase (AChE), présente dans les plaques séniles de la maladie d'Alzheimer, est résistante au bas pH, aux inhibiteurs et aux hautes concentrations de substrat. Les études cinétiques et pharmacologiques des fibres du complexe amyloïde-AChE indiquent que l'encombrement stérique de l'amyloïde au niveau de la gorge et du site périphérique de l'AChE est responsable de ces effets. (©Elsevier, Paris)

AChE / amyloid plaques / AChE-A β -amyloid fibril complexes / Alzheimer's disease

1. Introduction

Although Alzheimer's disease (AD) is a complex disorder with mutations in many genes accounting for 15–20% of AD patients, the large majority of AD cases occur sporadically [14]. There is therefore a need to search for the mechanisms responsible for the progressive cognitive decline observed in these cases. One of the most relevant neuropathological characteristics of AD brain is the senile plaques which are formed by a core of amyloid- β -peptide (A β) fibrils. Numerous other proteins have been found associated with amyloid deposits, including apolipoprotein E, α_1 -antichymotrypsin, heparan sulfate proteoglycans and acetylcholinesterase (AChE) among others [8, 13]. Most of the cortical AChE activity present in Alzheimers brain is predominantly associated with the amyloid core of senile plaques rather than with the neuritic component found at the periphery [9, 17]. Interestingly, histochemical studies indicate that the AChE associated with senile plaques differs enzymatically from the esterase associated with normal fibers and neurons in several respects [5].

A number of studies with synthetic A β in vitro have shown that this peptide aggregates and forms amyloid fibrils similar to the filaments found in the brains of AD patients [15]. The substitution of Glu22→Gln, found in hereditary cerebral hemorrhage with amyloidosis of the Dutch type, yields a peptide with increased ability to form amyloid fibrils [10]. On the other hand, the single mutation Val18→Ala induces a significant increment of the α -helical content of A β , and dramatically diminishes fibrillogenesis [16]. During the past years our labo-

ratory has shown that AChE promotes the assembly of A β _{1–40} peptide into amyloid fibrils, and that a hydrophobic environment close to the peripheral binding site of the enzyme is likely to be involved in this process [4, 7, 12]. Recently we found that stable AChE-A β complexes are formed during the growth of amyloid fibrils induced by AChE [1, 2, 11]. Here we examine the kinetic and pharmacological properties of AChE bound to amyloid fibrils formed with wild-type A β _{1–40} and two mutant peptides that differ in their fibrillogenic capacity: A β _{Val18→Ala} and A β _{Glu22→Gln}.

2. Enzymatic properties of the AChE present in AChE-A β complexes

Figure 1A, D, G shows the Lineweaver-Burk plots (1/v versus 1/S) obtained for free AChE and for the AChE bound in the following fibrillar complexes: AChE-A β _{1–40}, AChE-A β _{Val18→Ala} and AChE-A β _{Glu22→Gln}. It is clear that the kinetic parameters of the enzyme change, as the K_m and V_{max} values for AChE associated to amyloid were higher than those for the free enzyme in all three cases (see also table I). Comparison of the kinetic parameters given in table I shows that the K_m value for the AChE-A β _{1–40} complex was eight-fold higher than that for the enzyme alone. Similarly, for the AChE-A β _{Glu22→Gln} complex, the K_m value was elevated nine-fold and for AChE-A β _{Val18→Ala}, 28-fold. When kinetic studies were carried out under varying pH conditions, AChE associated to either the wild-type or the mutant A β peptides was more resistant to inhibition by low pH

Table I. Comparison of the kinetic parameters of acetylthiocholine hydrolysis obtained for the AChE associated with amyloid- β fibrils and the free enzyme.

Parameter	AChE	A β ₁₋₄₀ +AChE	A β _{Val-18→Ala} +AChE	A β _{Glu-22→Gln} +AChE
K_m (mM)	0.097 ± 0.007	0.764 ± 0.025*	2.680 ± 0.021*	0.856 ± 0.082*
V_{max} (μmol/mL/min)	0.129 ± 0.015	0.222 ± 0.029*	0.495 ± 0.018*	0.237 ± 0.025**

The AChE used in these studies was the hydrophobic G4 form affinity purified from bovine caudate nucleus on an acridine-Sepharose column, and the AChE associated with amyloid- β fibrils was obtained from the aggregation of the A β ₁₋₄₀ peptide induced by AChE, as described previously [1, 2, 7]. Values represent means ± S.D. of 4–6 separate assays made in duplicate for the AChE and 3–5 for the complex A β +AChE. The K_m and V_{max} were determined from Lineweaver-Burk plots (1/V vs. 1/S), with 0.03–1.0 mM acetylthiocholine. * Significantly different from control with $P < 0.001$ by non-paired Student's t -test; ** $P < 0.01$.

(Figure 1B, E, H). Similarly, AChE associated in AChE-A β complexes was more resistant to incubation under high substrate concentrations (figure 1C, F, I).

3. Pharmacological studies of the AChE present in AChE-A β complexes

The AChE associated to amyloid also appears more resistant to inhibition by anti-ChE agents as observed with both active site inhibitors such as tacrine, edrophonium and BW284c51, and with peripheral anionic site blockers, such as propidium and gallamine (table II). In almost all cases, a higher inhibitor concentration was required to obtain the same level of inhibition observed with the free enzyme. Overall, the A β ₁₋₄₀ analog containing the single amino acid substitution Val18 to Ala, showed the largest difference with respect to the free enzyme, suggesting that it has a greater degree of interaction with AChE than the other A β peptides. Contrastingly, the complex AChE-Dutch A β _{Glu22→Gln} mutant was the least affected of all the complexes studied, and in some cases, particularly for gallamine, almost no difference was found in the IC₅₀ values. In summary, for most of the anti-cholinesterases tested the inhibition of AChE associated to A β complexes ranked in the order: AChE-A β _{Val18→Ala} > AChE-A β ₁₋₄₀ > AChE-A β _{Glu22→Gln} > AChE.

These results are consistent with the idea that the association of AChE with A β fibrils leads to changes in its enzymatic properties, in the absence of any pathological alteration of the enzyme.

4. Discussion and conclusion

We have studied the kinetic and pharmacological properties of AChE incorporated into amyloid fibrils formed from the wild-type A β ₁₋₄₀ peptide as well as the mutants A β _{Val18→Ala} and A β _{Glu22→Gln}, and compared those characteristics with the free enzyme. Our results indicate that independently of the type of AChE-A β complex formed, the behavior of the bound enzyme differs to that of the free enzyme, and is reminiscent of the properties observed for the AChE present in the amyloid deposits of Alzheimer's brain. In previous work, the finding that the AChE associated to senile plaques presented lower sensitivity to excess substrate and to different anti-cholinesterase agents as well as higher resistance to inactivation by low pH than the normal neuronal enzyme [5], led to the suggestion that the enzyme bound to amyloid could correspond to a different molecular form of AChE induced during the disease [8]. Partial support for this hypothesis came from studies with the embryonic form of brain AChE, which also presents kinetic and pharmacological changes with respect to the adult enzyme [3]. These changes are similar to those observed for AChE bound to amyloid deposits. However, it is clear from the studies reported here that the same enzyme in two different environments can undergo kinetic and pharmacological modifications, and that the bound state of the enzyme is sufficient to induce these changes.

A plausible explanation for the differences observed is related to a physical phenomenon, i.e., the location or occlusion of the enzyme enmeshed within a fibrillar environment, in such a way as to produce

Figure 1. Change in the kinetic parameters of the AChE associated with amyloid- β fibrils. First row: Lineweaver-Burk (double-reciprocal) plots of AChE activity free and associated with amyloid- β fibrils over a range of substrate (ATCh) concentrations. **A.** Complex with wild-type A β . **D.** Complex with A β _{Val18→Ala}. **G.** Complex with A β _{Glu22→Gln}. Second row: pH dependence of the AChE free and associated with amyloid- β fibrils. Optimal pH of AChE and complexes was determined over a pH range of 4.0–9.0 in phosphate buffer. **B.** Complex with wild-type A β . **E.** Complex with A β _{Val18→Ala}. **H.** Complex with A β _{Glu22→Gln}. Third row: Activity of AChE as a function of substrate concentration. The rate of hydrolysis is plotted as a function of substrate concentration on a Log acetylthiocholine concentration. The bell-shaped curves show that AChE and the A β +AChE complex are subject to excess substrate inhibition but the free AChE is inhibited at low ATCh concentrations and the A β +AChE complex at higher ATCh concentrations. **C.** Complex with wild-type A β . **F.** Complex with A β _{Val18→Ala}. **I.** Complex with A β _{Glu22→Gln}.



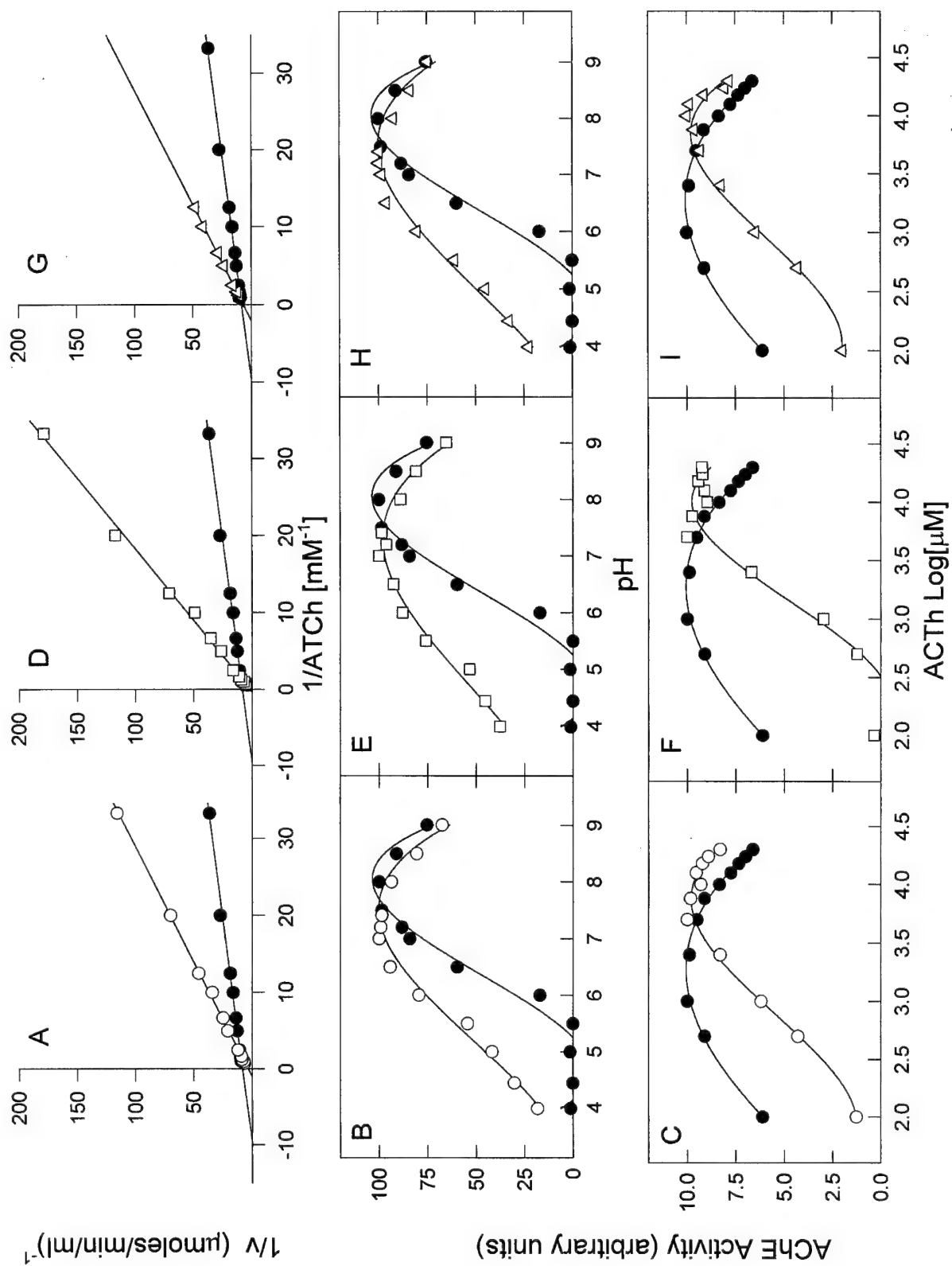


Table II. Effects of anti-ChE agents (IC₅₀) on the AChE associated with amyloid- β fibrils and the free enzyme.

Inhibitor	AChE	A β ₁₋₄₀ +AChE	A β _{Val-18→Ala} +AChE	A β _{Glu-22→Gln} +AChE
<i>Active site IC₅₀</i>				
Tacrine (10 ⁻⁹ M)	445.0 ± 17.6	1074.8 ± 73.3***	1422.6 ± 204.9***	625.8 ± 28.2***
Edrophonium (10 ⁻⁶ M)	5.36 ± 0.48	17.6 ± 4.6**	26.9 ± 0.2***	8.70 ± 0.92*
BW284c51 (10 ⁻⁹ M)	57.1 ± 1.3	116.9 ± 2.7***	193.2 ± 22.0***	91.5 ± 11.3**
<i>Peripheral site IC₅₀</i>				
Propidium (10 ⁻⁶ M)	34.6 ± 1.2	72.0 ± 4.8***	257.1 ± 24.0***	47.2 ± 2.1**
Gallamine (10 ⁻³ M)	8.76 ± 0.46	13.3 ± 0.45***	27.0 ± 2.5***	8.77 ± 0.97 ^{ns}

For determination of IC₅₀ values, samples were incubated with appropriate concentrations of the various inhibitors for 30 min at room temperature and the AChE activity was determined colorimetrically. Values represent means ± S.D. of 4–6 separate assay made in duplicate for the AChE and 3–5 for the complex A β +AChE. ns = not significant with $P > 0.05$ and significantly different from control with: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ by non-paired Student's t -test.

some degree of steric hindrance over the catalytic gorge and the peripheral binding site of the enzyme. Under these conditions, accessibility to the corresponding sites increases only after a reasonable increase in the substrate or inhibitor concentrations thereby giving rise to the effects on AChE activity seen for each specific compound.

Acknowledgments

This work was supported by grants from FONDECYT 1971240 and by a Presidential Chair in Science from the Chilean Government to N.C.I.

References

- [1] Alvarez A., Opazo C., Alarcón R., Garrido J., Inestrosa N.C., Acetylcholinesterase promotes the aggregation of amyloid- β -peptide fragments by forming a complex with the growing fibrils, *J. Mol. Biol.* 272 (1997) 348–361.
- [2] Alvarez A., Alarcón R., Opazo C., Campos E.O., Muñoz F.J., Calderón F.H., Dajas F., Gentry M.K., Doctor B.P., DeMello F.G., Inestrosa N.C., Stable complexes involving acetylcholinesterase and amyloid- β -peptide change the biochemical properties of the enzyme and increase the neurotoxicity of Alzheimers fibrils, *J. Neurosci.* 18 (1998) 3213–3223.
- [3] Arendt T., Brückner M.K., Lange M., Bigl V., Changes in acetylcholinesterase and butyrylcholinesterase in Alzheimers disease resemble embryonic development. A study of molecular forms, *Neurochem. Int.* 21 (1992) 381–396.
- [4] Campos E.O., Alvarez A., Inestrosa N.C., Brain acetylcholinesterase promotes amyloid- β -peptide aggregation but does not hydrolyze amyloid precursor protein peptides, *Neurochem. Res.* 23 (1998) 135–140.
- [5] Geula C., Mesulam M.M., Cholinergic systems and related neuropathological predilection patterns in Alzheimers disease, in: Terry R.D., Katzman R., Bick K.L. (Eds.), *Alzheimer Disease*, Raven Press, New York, 1994, pp. 263–291.
- [6] Inestrosa N.C., Alvarez A., Garrido J., Calderón F., Bronfman F.C., Dajas F., Gentry M.K., Doctor B.P. Acetylcholinesterase promotes Alzheimers β -amyloid fibril formation, in: Iqbal K., Winblad B., Nishimura T., Takeda M., Wisniewski H.M. (Eds.), *Alzheimers disease: biology, diagnosis and therapeutics*, John Wiley & Sons, London, 1997, pp. 501–510.
- [7] Inestrosa N.C., Alvarez A., Pérez C.A., Moreno R.D., Vicente M., Linker C., Casanueva O.I., Soto C., Garrido J., Acetylcholinesterase accelerates assembly of amyloid- β -peptides into Alzheimers fibrils: possible role of the peripheral site of the enzyme, *Neuron* 16 (1996) 881–891.
- [8] Kalaria R.N., Kroon S.N., Grahovac I., Perry G., Acetylcholinesterase and its association with heparan sulphate proteoglycans in cortical amyloid deposits of Alzheimer's disease, *Neuroscience* 51 (1992) 177–184.
- [9] Kasa P., Rakonczay Z., Gulya K., The cholinergic system in Alzheimer's disease, *Prog. Neurobiol.* 52 (1997) 511–535.
- [10] Levy E., Carman M., Fernández-Madrid I., Power M., Lieberburg I., van Duinen S., Gerard T., Bots A., Luyendijk W., Frangione B., Mutation of the Alzheimers disease amyloid gene in hereditary cerebral hemorrhage, Dutch type, *Science* 248 (1990) 1124–1128.
- [11] Opazo C., Inestrosa N.C., Crosslinking of amyloid- β peptide to brain acetylcholinesterase, *Mol. Chem. Neuropathol.* 33 (1998) 39–49.
- [12] Reyes A.E., Perez D.R., Alvarez A., Garrido J., Gentry M.K., Doctor B.P., Inestrosa N.C., A monoclonal antibody against acetylcholinesterase inhibits the formation of amyloid fibrils induced by the enzyme, *Biochem. Biophys. Res. Commun.* 232 (1997) 652–655.
- [13] Selkoe D.J., Amyloid β -protein and the genetics of Alzheimers disease, *J. Biol. Chem.* 271 (1996) 18295–18298.
- [14] Selkoe D.J., Alzheimers disease: genotypes, phenotype, and treatments, *Science* 275 (1997) 630–631.
- [15] Soto C., Brañes M.C., Alvarez J., Inestrosa N.C., Structural determinants of the Alzheimers amyloid β -peptide, *J. Neurochem.* 63 (1994) 1191–1196.
- [16] Soto C., Castaño E., Frangione B., Inestrosa N.C., The α -helical to β -strand transition in the N-terminal fragment of the amyloid β -peptide modulates amyloid formation, *J. Biol. Chem.* 270 (1995) 3063–3067.
- [17] Ulrich J., Meier-Ruge W., Probst A., Meier E., Ipsen S., Senile plaques: staining for acetylcholinesterase and A4 protein: A comparative study in the hippocampus and entorhinal cortex, *Acta Neuropathol.* 80 (1990) 624–628.

Cholinergic modulation of extracellular ATP-induced cytoplasmic calcium concentrations in cochlear outer hair cells

Martin A. Wikström^a, Grace Lawoko^b, Edith Heilbronn^{b*}

^a*Nobel institute for Neurophysiology, Department of Neuroscience, Karolinska institutet, S-171 77 Stockholm, Sweden*

^b*Department of Neurochemistry and Neurotoxicology, Stockholm University, S-106 91 Stockholm, Sweden*

Abstract — Outer hair cells (OHC) of the mammalian cochlea modulate the inner hair cell (IHC) mechanoelectrical transduction of sound. They are contacted by synapsing efferent neurons from the CNS, their main efferent neurotransmitter being acetylcholine (ACh). OHC function and in particular their control of $[Ca^{2+}]_i$ is highly important and is modulated by ACh and also by other substances including extracellular (EC) ATP. OHC carry at their efferent synapse a not yet completely identified neuronal type of ionotropic ACh receptor (AChR), with an unusual pharmacology, which is, in vivo and in vitro, reversibly blocked by α -bungarotoxin (α -bgtx). The AChR mediates a fast influx of Ca^{2+} into OHC which, in turn, activates a closely located outwardly-directed Ca^{2+} -dependent K^+ -channel, thus shortly hyperpolarizing the cell. A cloned homomeric $\alpha 9$ nAChR mimicks the function and pharmacology of this receptor. We here report results from a study designed to observe only slower effects triggered by EC ATP and the ACh-AChR system. EC presence of ATP at OHC increases $[Ca^{2+}]_i$ by activating both P_{2x} and P_{2y} purinoceptors and also by indirect activation of OHC L-type Ca^{2+} -channels. The L-type channel activation is responsible for a large part of the $[Ca^{2+}]_i$ increase. Simultaneous EC presence of ACh and ATP at OHC was found to depress ATP-induced effects on OHC $[Ca^{2+}]_i$, an effect that is completely blocked in the presence of α -bgtx. Our observations suggest that the ACh-AChR system is involved in the modulation of the observed EC ATP-triggered events; possibly the OHC AChR is able to act both in its well known rapid ionotropic way, but also, perhaps after modification in a slower, metabotropic way interfering with the EC ATP-induced $[Ca^{2+}]_i$ increase. (©Elsevier, Paris)

Résumé — Modulation cholinergique de la concentration de Ca^{2+} cytoplasmique induite par l'ATP extracellulaire dans les cellules ciliées de la cochlée. Nos observations suggèrent que le système ACh-RACH est impliqué dans la modulation des événements induits par l'ATP extracellulaire ; il est possible que les RACH des cellules ciliées soient capables d'agir à la fois par la voie rapide ionotrope mais aussi, peut-être, par une modification d'une voie métabotrope plus lente interférant avec l'augmentation de $[Ca^{2+}]_i$ intracellulaire induite par l'ATP extracellulaire. (©Elsevier, Paris)

outer hair cell / purinergic / muscarinic-cholinergic / modulation / calcium

1. Introduction

The apparatus responsible for the transduction of sound energy into neural activity is the organ of Corti, located within the cochlear duct. The sensory cells, one row of inner haircells (IHC) and three rows of outer haircells (OHC) are attached at their basal end to the basilar membrane while only the stereocilia of the OHC apical end are embedded into the tectorial membrane; those of IHC reach but do not quite touch it [5, 9]. IHC act as mechanoelectrical transducers and release neurotransmitter onto neurons of the spiral ganglion inside the cochlea, thus initiating, via the cochlear component of the cranial nerve VIII (see e.g. [8]), the conduction of auditory information into the brain and the auditory cortex. Information about acoustic signals to the central nervous system (CNS) passes via nuclei in the me-

dulla, pons, midbrain and thalamus to the auditory cortex. 95% of the afferent fibers contact IHC and only 5% OHC.

The role of OHCs is interesting; they are part of a feedback system from the CNS and believed to take part in the control of the sensitivity of IHC and the primary afferent nerve to a specific acoustic stimulus [4, 14]. OHC appear to be able to modulate their shape, i.e., to shorten when they depolarize and to lengthen when they hyperpolarize in response to sound stimulus, an ability that, by increasing or decreasing tension on the tectorial membrane, is believed to allow the cells to influence the transfer of sound energy to the IHC.

Efferent innervation of OHC mainly consists of medial olivocochlear fibers that use acetylcholine (ACh) as their main transmitter [8]. Other substances influence the signal from the nerve to OHC; adenosine 5'-triphosphate (ATP), believed to act as a modulator on OHC, γ -aminobutyric acid (relevant for more apical OHC) and, for example, enkephalins.

* Correspondence and reprints

It is well established that the efferent synapse of OHC carries a presynaptic muscarinic acetylcholine receptor (mAChR) subtype [3] probably involved in transmitter release, of which so far little is known. Postsynaptically, an ionotropic, reversibly α -bungarotoxin (α -bgtx)-binding [1, 10, 15, 17] neuronal type of AChR was described. This AChR allows a very rapid Ca^{2+} -influx into OHC which in turn mediates the activation of a closely located Ca^{2+} -dependent, outwardly directed K^{+} -channel which hyperpolarizes the cell [12, 13]. The OHC AChR is classified as a neuronal nicotinic nAChR ($\alpha 9$) subtype with unusual properties [6, 7], i.e., it has a mixed nicotinic-muscarinic pharmacology and is sensitive to strychnine [7, 13, 15, 17].

OHC also carry both ionotropic ($\text{P}_{2\text{X}}\text{R}$) and metabotropic ($\text{P}_{2\text{Y}}\text{R}$) purinergic receptors ([11, 16, 18] and others) and extracellular (EC) ATP was shown early this decennium to act as an important modulator on OHC [16]. The activation of these receptors strongly increases intracellular $[\text{Ca}^{2+}]_{\text{i}}$, from respectively extra- and intracellular sources [2] and so do L-type Ca^{2+} -channels seemingly activated as a secondary event after EC ATP-application [16].

The purinergic and the cholinergic systems appear to interact. Simultaneous EC presence of ACh and ATP depresses the $[\text{Ca}^{2+}]_{\text{i}}$ increases induced in OHC as compared to those induced by EC ATP alone (for review see [11]). Here we present results from a study of mechanisms involved in the control of OHC $[\text{Ca}^{2+}]_{\text{i}}$ by the two compounds, their receptors and the L-type Ca^{2+} -channel, including observations pointing to the AChR located at the OHC efferent synapse as being able to act both as an ionotropic and a metabotropic AChR.

2. Materials and methods

2.1. Preparation of outer hair cells

Pigmented guinea pigs of either sex (Duncin Hartley) were anaesthetized with CO_2 prior to decapitation. OHC were non-enzymatically microdissected as previously described in [15]. The temporal bones were removed and dissected in ice-cold Hanks balanced salt solution (HBSS) containing (in mM): NaCl 137, KCl 5.37, MgSO_4 0.81, KH_2PO_4 0.4, CaCl_2 1.25, Na_2HPO_4 0.35, NaHCO_3 4.2, D-glucose 5.5, Hepes 5.0 (pH 7.4, osmolarity, 320 ± 3 mOsm/L). OHC's were then separated from membranes and supporting cells by gentle pipetting. Only OHC from the second and third turns of the cochlea were used for experiments.

2.2. Calcium imaging

Isolated OHC were loaded with fluo-3/AM (2 mM) for 30 min at 20 – 22°C in HBSS. After incubation, 300 μL of the cell

suspension was transferred onto concanavalin A-coated coverslips which formed the bottom of an open perfusion chamber (volume, 0.1 cm^3). The perfusion rate was approximately 100 $\mu\text{L}/\text{min}$ and the chamber was placed on the stage of an inverted Leitz fluovert FU microscope.

A confocal laser scanning fluorescence microscope (CLSM-Leica, Germany) with an Argon-Krypton laser (Coherent) using the 488 nm line was used for measurements of the relative cytoplasmic calcium concentration ($[\text{Ca}^{2+}]_{\text{i}}$) in OHC's. The fluo-3 loaded cells were excited at 488 nm and emitted fluorescence was passed through a 515 nm long pass filter and displayed. The time resolution of the measurement was 3.0 s. Results were expressed as the ratio of fluorescence/resting fluorescence, i.e., F/F_0 .

2.3. Electrophysiology

Conventional patch-clamp experiments in whole-cell and perforated patch configurations were carried out using an Axopatch 200 patch-clamp amplifier (Axon instruments, Foster City, USA). During experiments the cells were immersed in Leibkowitz medium (L-15, Gibco) containing (in mM): NaCl 135, KCl 4, CaCl_2 1.26, MgCl_2 2 and amino acids (pH 7.4, osmolarity, 325 ± 2 mOsm/L). In a few cases HBSS was used instead. Pipettes were pulled from borosilicate glass on a DMZ universal puller and had a resistance between 3–6 M Ω . Pipettes for whole-cell recordings were back-filled with an intracellular solution containing (in mM): KCl 144, MgCl_2 4, EGTA 5, Na_2ATP 2, Na_2HPO_4 8, NaH_2PO_4 2, (pH 7.3) while in pipettes for perforated-patch experiments the solution used had a composition of (in mM): KCl 10, K_2SO_4 76, NaCl 10, MgCl_2 1, Hepes 10 (pH 7.35) and amphotericin B final concentration 0.1%. Experiments were carried out in current-clamp mode to monitor changes in membrane potential.

2.4. Chemicals

Acetylcholine chloride (ACh), adenosine 5' triphosphate (ATP), amphotericin B and concanavalin A (type IV) were obtained from Sigma (St. Louis, Missouri, USA); fluo-3/AM, D-600 and nifedipine were obtained from Molecular Probes (Europe BV, the Netherlands) and suramin monosodium salt was from Bayer (Leverkusen, Germany).

3. Results

Application of 10–100 μM EC ATP to OHC mediated a concentration-dependent and reversible increase in $[\text{Ca}^{2+}]_{\text{i}}$ (figure 1, $n = 29$) and a potent depolarization ($n = 22$). The increase of the $[\text{Ca}^{2+}]_{\text{i}}$ was blocked by application of the non-selective P_2 receptor antagonist suramin (50–100 μM) thereby demonstrating the presence of such receptors on OHC, confirming earlier results ([2] and others).

When OHC were immersed in Ca^{2+} -free extracellular solution, EC ATP (100 μM) was still able to elicit an increase of the $[\text{Ca}^{2+}]_{\text{i}}$ in most (figure 2, $n = 5$ of 8) cells although the amplitude was depressed. This demonstrates that the EC ATP-induced

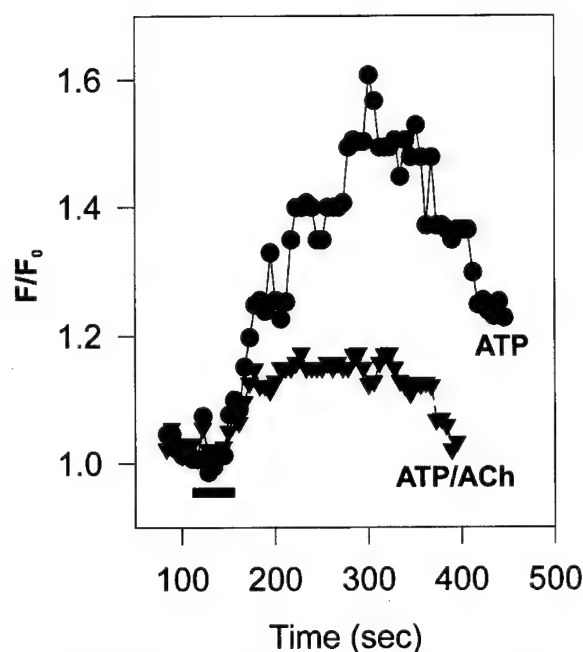


Figure 1. EC ATP (1–100 μ M) induces an increase in OHC $[Ca^{2+}]_i$ in a concentration-dependent manner. When ACh (25–100 μ M) is coapplied with ATP the amplitude of the $[Ca^{2+}]_i$ increase is markedly depressed. At even higher concentrations of ACh (up to 100 μ M) the depression got even more pronounced.

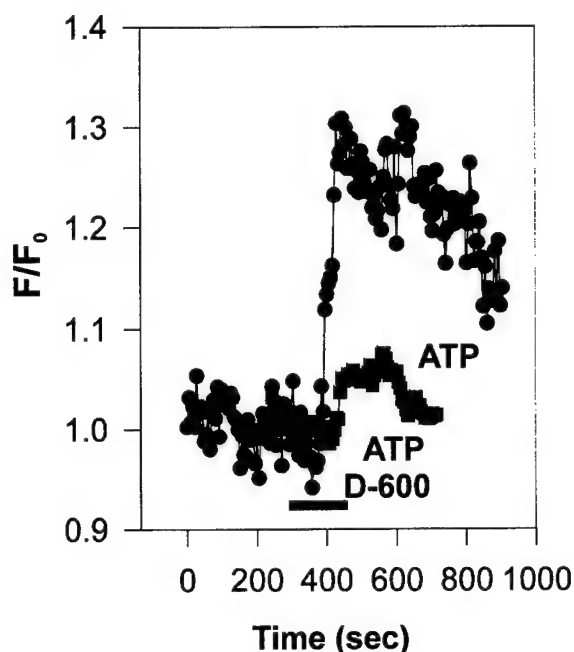


Figure 3. The increase in $[Ca^{2+}]_i$ induced by EC ATP (10–100 μ M) was suppressed in a concentration-dependent manner, when the L-type Ca^{2+} channel blocker D-600 (1–100 μ M) was present.

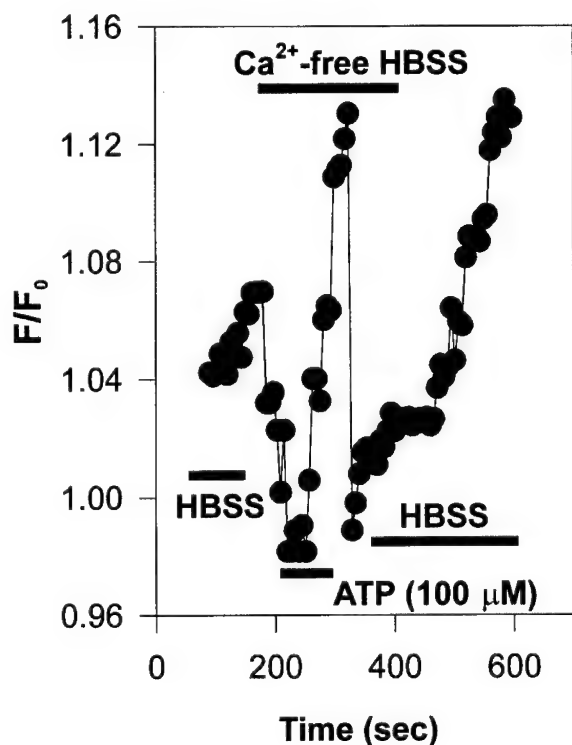


Figure 2. EC ATP (100 μ M) is able to increase the $[Ca^{2+}]_i$ in Ca^{2+} -free extracellular solution although the amplitude is depressed as compared to control.

$[Ca^{2+}]_i$ increase is mediated both by influx of Ca^{2+} into OHC as well as by release of Ca^{2+} from intracellular stores.

To investigate if L-type Ca^{2+} channels contribute to the increase in $[Ca^{2+}]_i$ induced by EC ATP application, we exposed OHC to the L-type Ca^{2+} channel blockers D-600 (1–100 μ M, $n = 15$), or nifedipine (2–5 μ M, $n = 4$) during EC ATP application. The $[Ca^{2+}]_i$ increase caused by application of EC ATP was strongly depressed (figure 3) in a concentration-dependent manner, demonstrating that these channels are activated and contribute to the rise in $[Ca^{2+}]_i$.

ACh (25–50 μ M) when applied to OHC before or during EC ATP-application (50 μ M) mediated a clear depression of the increase in $[Ca^{2+}]_i$ (figure 1, $n = 3$) compared to that induced by EC ATP alone. ACh alone had no effect on $[Ca^{2+}]_i$ or membrane potential at the time resolution used in these experiments ($n = 6$).

To investigate if ACh application modulates the $[Ca^{2+}]_i$ increases due to EC ATP through an action on L-type Ca^{2+} channels, experiments were performed in which L-type channels were blocked by D-600 (50 μ M, $n = 3$); the results show that even under these circumstances ACh was able to depress the increase in $[Ca^{2+}]_i$ induced by EC ATP.

During experiments performed in Ca^{2+} -free extracellular solution no increase in $[Ca^{2+}]_i$ was normally observed when ACh and ATP were co-applied ($n = 9$).

We furthermore investigated if α -bgtx, known to reversibly antagonize the fast AChR-induced Ca^{2+} influx into OHC and their subsequent hyperpolarization, also blocked the ACh-mediated depression of the EC ATP-induced $[Ca^{2+}]_i$ increase. When 50 nM of α -bgtx was present, application of 50 μ M of EC ATP generated a large increase in the $[Ca^{2+}]_i$ also in the presence of 50–100 μ M ACh (figure 4, $n = 3$). α -bgtx had no effect on the increase in $[Ca^{2+}]_i$ due to EC ATP only ($n = 3$).

4. Discussion

We have confirmed earlier observations that EC ATP activates P_2 receptors on the OHC membrane and that this results in a marked increase in $[Ca^{2+}]_i$. The cells were also potentially depolarized by EC ATP.

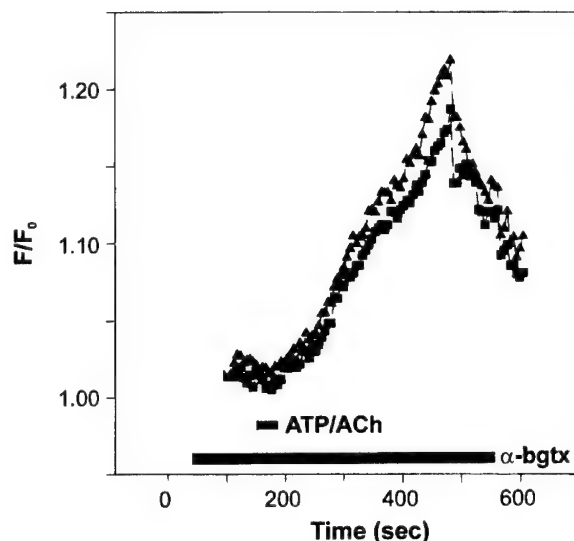


Figure 4. α -bgtx (50 nM) clearly antagonized the ACh mediated depression of the EC ATP-induced (100 μ M) $[Ca^{2+}]_i$ increase. At ACh concentrations (100 μ M) that normally almost blocked the $[Ca^{2+}]_i$ increase completely, a relatively large $[Ca^{2+}]_i$ was observed when α -bgtx was present. The two traces indicate different regions of the cell (triangles, middle; squares, basal).

The $[Ca^{2+}]_i$ increase induced by EC ATP was not abolished in Ca^{2+} -free extracellular solution which shows that the increase in $[Ca^{2+}]_i$ induced by EC ATP is due to both influx of Ca^{2+} into the cell via ionotropic P_{2x} receptors as well as release of Ca^{2+} from intracellular sources via the activation of P_{2y} receptors.

L-type Ca^{2+} channels have been shown to be present in OHC. When L-type channels were blocked with relevant blockers the EC ATP-induced $[Ca^{2+}]_i$ increase was strongly suppressed in a concentration-dependent manner. This demonstrates that these voltage-dependent channels are activated during EC ATP application, probably as a result of the initial depolarization of the cell, and strongly contribute to the ATP-induced $[Ca^{2+}]_i$ increase.

ACh suppresses the increase in $[Ca^{2+}]_i$ induced by EC ATP, a known but unexplained phenomenon reconfirmed here. We found that ACh depressed the remaining EC ATP-induced $[Ca^{2+}]_i$ increase in a similar proportion also when L-type Ca^{2+} channels were blocked. We therefore suggest that L-type Ca^{2+} channels are not directly involved in the mechanism for the ACh-induced depression of the EC ATP-induced $[Ca^{2+}]_i$ increase. Furthermore, in Ca^{2+} -free extracellular solution no increase in $[Ca^{2+}]_i$ was normally observed when ACh and ATP were co-applied, suggesting that ACh might modulate the EC ATP-induced $[Ca^{2+}]_i$ increase, at least partly, through an effect on the P_{2y} receptor mediated release on Ca^{2+} from intracellular stores.

α -bgtx, a reversible antagonist at the OHC ionotropic AChR, was also able to antagonize the ACh-mediated depression of the EC ATP-induced increase in $[Ca^{2+}]_i$, suggesting that this receptor might have a dual function. On a very short timescale the receptor acts ionotropically, allowing rapid influx of Ca^{2+} resulting in a short-lasting hyperpolarization of OHC due to the activation of the outwardly directed Ca^{2+} -activated K^+ channels. On a timescale of seconds to minutes the ACh-AChR system does not affect OHC $[Ca^{2+}]_i$ significantly but it modulates $[Ca^{2+}]_i$ increases mediated by EC ATP application. This latter phenomenon may be a metabotropic effect.

In summary the results suggest that the basolaterally located AChR of OHC may have both metabotropic and ionotropic properties. The mechanism for this is at present unknown. Possibly, a phosphorylation of the AChR on the external membrane is required for the receptor to function in a metabotropic manner. Alternatively the 'unmodified' AChR itself can act in this way. Its structure is still not completely known. The possibility that OHC instead express two different AChR remains, but this seems

unlikely as the pharmacology of the rapid and the slow AChR-induced actions seems to be identical.

References

- [1] Anniko M., Stenqvist M., Pettersson A., Heilbronn E., Alpha-bungarotoxin inhibits outer hair cell motility in situ. *J. Otorhinolaryngol.* 57 (1995) 105–109.
- [2] Ashmore J.F., Ohmori H., Control of intracellular calcium by ATP in isolated outer hair cells of guinea pig cochlea, *J. Physiol. (Lond.)* 428 (1990) 109–131.
- [3] Bartolami S., Ripoll C., Planche M., Pujol R., Localisation of functional muscarinic receptors in the rat cochlea: evidence for efferent presynaptic autoreceptors, *Brain Res.* 626 (1993) 200–209.
- [4] Brownell W.E., Bader C.R., Bertrand D., Ribaupierre Y.D., Evoked mechanical responses of isolated cochlear outer hair cells, *Science* 227 (1985) 194–196.
- [5] Delcomyn F., Foundations of neurobiology, W.H. Freeman and company, New York, 1997.
- [6] Elgoyhen A.B., Johnson D., Boulter J., Vetter D., Heinemann S., Alpha 9: An acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells, *Cell* 79 (1994) 705–715.
- [7] Erostequi C., Norris C.H., Bobbin R.P., In vitro characterization of a cholinergic receptor on outer hair cells, *Hear Res.* 74 (1994) 135–147.
- [8] Eybalin M., Neurotransmitters and neuromodulators of the mammalian cochlea, *Physiol. Rev.* 73 (1993) 309–372.
- [9] Ganong W.F., Hearing and equilibrium, in: *Review of Medical Physiology*, Prentice Hall International, 1991 pp. 158–170.
- [10] Guth P.S., Norris C.H., The hair cell acetylcholine receptors: a synthesis, *Hearing Res.* 98 (1996) 1–8.
- [11] Heilbronn E., Järleback L., Lawoko G., Cholinergic and purinergic signalling in outer hair cells of mammalian cochlea, *Neurochem. Int.* 27 (1995) 301–311.
- [12] Housley G.D., Ashmore J.F., Direct measurement of the action of acetylcholine on isolated outer hair cells of the guinea pig cochlea, *Proc. Roy. Soc. Lond. [Biol.]* 244 (1991) 161–167.
- [13] Kakehata S., Nakagawa T., Takasaka T., Akaike N., Cellular mechanism of acetylcholine-induced response in dissociated outer hair cells of guinea-pig cochlea, *J. Physiol. (Lond.)* 463 (1993) 227–244.
- [14] Kelly J.P., Hearing, in: Kandel E.R., Schwartz J.H., Jessell T.M. (Eds.), *Principles of neural science*, Elsevier, New York, 1991, pp. 481–499.
- [15] Lawoko G., Järleback L., Heilbronn E., Ligand binding properties of an unusual nicotinic acetylcholine receptor subtype on isolated outer hair cells from guinea pig cochlea, *Neurosci. Lett.* 195 (1995) 64–68.
- [16] Nilles R., Järleback L., Zenner H.P., Heilbronn E., ATP-induced cytoplasmic $[Ca^{2+}]$ increases in isolated cochlear outer hair cells. Involved receptor and channel mechanisms, *Hear. Res.* 73 (1994) 27–34.
- [17] Plinkert P.K., Zenner H.P., Heilbronn E., A nicotinic acetylcholine receptor-like α -bungarotoxin-binding site on outer hair cells, *Hear. Res.* 53 (1991) 123–130.
- [18] Raybould N.P., Housley G.D., Variation in expression of the outer hair cell P_{2X} receptor conductance along the guinea-pig cochlea, *J. Physiol. (Lond.)* 498 (1997) 717–727.

The acetylcholine, GABA, glutamate triangle in the rat forebrain

Giancarlo Pepeu, Patrizio Blandina

Department of Preclinical and Clinical Pharmacology, University of Florence, Viale G.B. Morgagni 65, 50134 Florence, Italy

Abstract — The present overview demonstrates that stress, fear, novelty, and learning processes are associated with arousal and increases of extracellular levels of cortical and hippocampal ACh, independently of increases of motor activity. Forebrain cholinergic systems appear to be regulated by GABAergic and glutamatergic inputs. However, several other neurotransmitter systems play a role. (©Elsevier, Paris)

Résumé — Le triangle acétylcholine, GABA, glutamate dans le cerveau antérieur du rat. Nous résumons ici un ensemble de résultats qui démontrent que le stress, la peur, la nouveauté, et les processus d'apprentissage sont associés à l'éveil et à une augmentation des niveaux d'acétylcholine extracellulaire dans l'hippocampe et le cortex, indépendamment de l'augmentation d'activité motrice. Le système cholinergique du cerveau antérieur semble être contrôlé par l'innervation GABAergique et glutamatergique. Cependant, plusieurs autres systèmes de neurotransmetteurs interviennent également. (©Elsevier, Paris)

acetylcholine / GABA / glutamate / microdialysis

1. Cognitive processes associated with a raise in extracellular ach levels

The roles of the forebrain cholinergic systems in the cognitive functions have been reviewed recently [16], and the following functions have been identified: a) the nucleus basalis magnocellularis (NBM)-cortical cholinergic system contributes greatly to visual attentional functions, but not to mnemonic processes per se; b) the septo-hippocampal cholinergic projection is involved in the modulation of spatial working memory processes; and c) the diagonal band-cingulate cortex cholinergic projection impacts on the ability to utilize response rules through conditional discrimination.

The activity of the cholinergic systems ascending from the NBM to the cortex, and from the septum to the hippocampus can be monitored in vivo measuring the extracellular levels of acetylcholine (ACh) by microdialysis. Rat exposure to novel environment evoked an exploratory behavior involving increase of attention and locomotor activity, and elicited large increases of extracellular level of cortical ACh [21, 29], which faded with habituation to the environment. Rats performing spontaneous alternation in a Y maze also displayed a large increase in extracellular level of cortical ACh [21]. This task requires attention, and involves spatial working memory and motor activity. Conversely, object exploration non-associated with increase of locomotor activity failed to alter extracellular levels of cortical ACh, although this task is known to depend on the intact cortical cholinergic system [4, 15]. Novelty appears to acti-

vate also the septo-hippocampal system as shown by the increase of ACh extracellular level in dorsal hippocampus of rats introduced in an open field, thus inducing an exploratory activity associated with increase of locomotion [3]. Indeed, the increase in ACh extracellular level strictly matched the intensity of locomotor/exploratory activity. Object exploration, on the other hand, elicited a much smaller increase of extracellular levels of ACh than novelty (Aloisi, personal communication). Although these experiments show a relationship between motor and cholinergic activities, it is not yet clear whether the increases of ACh release elicited by exposure to novel environment are associated with, or depend on locomotor activity. Indeed, locomotor activation correlated with increases of ACh release in the hippocampus, cortex and striatum of adult rats [12], and in the frontal cortex of young but not of old rats [34]. Moreover, Nilsson and Björklund [37] reported that a short period of swimming was associated with a sharp increase of hippocampal ACh extracellular levels. This increase, however, persisted for a long time after the swimming session, thus indicating that it was a consequence of the arousal induced by swimming more than that of the locomotor activity. Accordingly, novelty represented not by the environment but by unconditioned stimuli, such as a tone or a light, caused a large increase of ACh extracellular levels in the frontal cortex and the hippocampus but little motor activity [1], and fear related behaviors, among which freezing is predominant, are also characterized by large increases of extracellular ACh levels in both cortex and hippocampus.

Although both trained and untrained rat groups showed similar motor activity when placed in an operant chamber, only untrained rats exhibited increases of ACh extracellular levels of frontal cortex and hippocampus associated with a rise in the reinforced responses, i.e., when the rats were acquiring the procedure [38]. Thus, the performance of the trained rats did not activate the forebrain cholinergic systems. Other studies, however, reported that cortical ACh extracellular levels increased during reinforced response execution also in well trained rabbits [40], but the paradigm used involved response inhibition, which requires attention and arousal. A very large increase of ACh levels was reported in the frontal cortex of trained rats during anticipation of a palatable meal, a stimulus associated with strong arousal [43]. Finally, the increase of extracellular levels of rat hippocampal ACh was much larger during acquisition of a discriminative task than during that of a non-discriminative task [48].

Therefore, attention and arousal appear to be associated with the activation of both cortical and hippocampal cholinergic systems, independently from an increase of motor activity. Consistently, presentation of sensory stimuli elicited an increase in ACh levels of the frontal cortex and hippocampus without changes in motor activity [2, 28]. Ragozzino et al. [39] reported that intrahippocampal infusion of glucose failed to modify ACh spontaneous release, but enhanced hippocampal ACh output during spontaneous alternation, and improved the performance, thus confirming the role of hippocampal ACh in this task, which involves attention and spatial working memory.

Also, stress activates the forebrain cholinergic pathways, likely because of its association with arousal. Restraint stress induced in male rats a marked increase of ACh release from the hippocampus [26, 27, 37, 51]. A further increase of hippocampal ACh levels was observed immediately after the rats were set free [46]. Novelty represents a stressful condition for the rat, and the cholinergic activation caused by exposure to novel environment is attributable, at least partly, to stress. Handling, which is a mixture of stress and sensory stimulation, also caused an increase of hippocampal ACh levels [36, 37], but gentle handling used to move rats from a cage and to put them back immediately failed to alter cortical ACh levels [21]. Thus, it seems likely that while an increase in locomotor activity is associated with an increase in ACh release from the cerebral cortex and hippocampus, there are conditions, such as stress, fear, novelty, learning process, in which the activation of the cholinergic systems occurs independently from the increase in motor activity.

2. Which mechanisms activate the forebrain cholinergic neurons?

Regardless of whether the forebrain cholinergic neurons are activated by sensory stimuli, stress, novelty or learning, the question arises as to the nature of the signal triggering them, thus leading to increased firing and, consequently, increase of ACh release.

2.1. Septal-hippocampal pathway

GABA is largely present in the basal forebrain, septum, hippocampus, and cerebral cortex [45], where it appears to play an important role in the modulation of cholinergic neurones. GABAergic inputs from lateral to medial septum impinge on cholinergic neurons [32]. Intraseptal administration of muscimol, a GABA_A receptor agonist, decreased both release [19, 24], and turnover [5] of hippocampal ACh. Intraseptal administration of bicuculline, a GABA_A receptor antagonist, increased the hippocampal rate of high-affinity choline uptake [50], thus suggesting that GABA exerts a tonic inhibition of the cholinergic activity [14].

If septal cholinergic neurons are controlled by GABAergic neurons, the next question is: how are those latter regulated? Glutamate seems to play an important role. Glutamatergic inputs to the septum exerted a tonic excitatory influence on septal GABAergic activity [19], thus suggesting that they might inhibit indirectly septal-hippocampal cholinergic neurons. Indeed, intraseptal administration of 3-(2 carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP), an NMDA receptor antagonist, reduced GABA extracellular level in the septum, simultaneously increasing hippocampal ACh release [19]. There is no evidence that glutamate influence directly septal cholinergic neurons. The tonic regulation of septal-hippocampal cholinergic activity by dopamine appears also to be mediated by GABA, since dopamine effects on hippocampal cholinergic activity are reversed by bicuculline [13].

2.2. NBM-cortical pathway

GABA affects the cortical cholinergic activity at three levels: the NBM, the septum, and the cortex. Direct injection of muscimol into NBM decreased cortical ACh release. This effect was blocked by picrotoxin, a GABA_A antagonist [8]. Moreover, cortical ACh output is decreased by the infusion into the basal forebrain of benzodiazepine receptor agonists, and is enhanced by its antagonists [36]. Finally, while systemic administration of picrotoxin increased ACh spontaneous release from the frontal cortex,

diazepam injected i.p. reduced the increase of ACh induced by tactile stimulation [2]. Local administration of CPP into the septum increased cortical ACh and GABA release, and the effect on ACh was prevented by muscimol [20]. In the medial septum the entire population of neurons is represented by cholinergic and GABAergic cells [30]. GABA neurons project from the medial septum to the cortex [20], thus suggesting that septal-cortical GABAergic neurons modulate cortical cholinergic activity. These GABA neurons, in turn, appear to be regulated by glutamatergic inputs to the GABAergic interneurons of the lateral septum. Indeed, the blockade of NMDA receptors located on these GABAergic interneurons increased the release of cortical GABA [20]. It is not yet clear by which mechanism GABA elicits an increase of ACh release from the cholinergic nerve endings. At least two explanations can be offered. First, the activation of a GABA transporter localized on cholinergic terminals, which appears to regulate also ACh release [7]. Alternatively, the increase in cortical GABA levels could inhibit GABAergic interneurons, which in turn inhibit ACh release. While local administration of GABA did not affect spontaneous ACh release from the cerebral cortex of freely moving rats, perfusion with bicuculline caused a concentration-dependent increase of ACh release. The effect of bicuculline is blocked by muscimol [17]. These findings confirm that the cortical cholinergic network is under a tonic inhibitory GABAergic control mediated by GABA_A receptors. GABA also appears to regulate the release of cortical ACh through a pre-synaptic action, and some 5-HT/ACh interactions may be mediated by GABA [22]. Moreover, through the activation of cortical histamine H₃ receptors, histamine has been shown to inhibit ACh release from rat cortex in vivo [6] through cortical GABA interneurons [18]. Finally, the GABAergic fibers ascending from the septum could impinge on, and modulate the activity of cortical intrinsic cholinergic neurons. Their existence in the rat brain has been recently confirmed by immuno-histochemistry of the vesicular ACh transporter [44]. However, their functional role, and their contribution to cortical ACh extracellular levels need to be defined.

The glutamatergic pathways modulate the cortical cholinergic activity both indirectly, regulating the activity of the GABAergic neurons, and directly, impinging on the cholinergic neurons of the NBM. Indeed, the perfusion of NBM with CPP [20], or kynurenate [41] decreased cortical ACh spontaneous release. Moreover, the cortical ACh release induced by brain stem stimulation is inhibited also by kynurenate application to the NBM [41]. This observation indicates that a major glutamatergic pathway, stimulating the cholinergic neurons of the NBM through

NMDA receptors, originates from the pedunculopontine tegmentum [42]. According to these authors, AMPA receptors are only involved in the desynchronization, presumably through a circuit including GABAergic projections to the thalamus.

3. Conclusions

The forebrain cholinergic systems are activated in conditions inducing arousal, whether or not associated with an increase in motor activity. The arousal may be induced by non-cognitive stimuli, such as stress or sensory stimulation, or by performance of tasks involving learning and working memory, such as exploration of a novel environment, spontaneous alternation, acquisition of a behavior. The neuronal circuits and neuro-transmitters which trigger the activation of the forebrain cholinergic systems during arousal are not entirely clear yet. The interactions of cholinergic neurons located in the septum and NBM with GABAergic and glutamatergic systems here reviewed, suggest that GABAergic interneurons exert an inhibitory tone on cholinergic neurons. However, this mechanism is not universal, since in the cerebral cortex increases of GABA release are associated with those of ACh release [20]. In the dorsal hippocampus, the local stimulation of non-NMDA receptors stimulates the release of both GABA and ACh through a mechanism occurring at nerve ending level [23]. Further studies will be necessary to learn the behavioral implications of the interactions between cholinergic and GABAergic systems, including experiments correlating changes of ACh outflow in behaving rats with those of GABA extracellular levels. The GABAergic neurons are in turn modulated by many neurotransmitters, including glutamate. However, it has been shown that glutamatergic neurons affect the cholinergic neurons not only indirectly, modulating the GABAergic tone, but also directly. With the caveat of the dual role of neurotransmitter and metabolite of glutamate, investigations correlating changes in glutamate, GABA and ACh extracellular levels during specific behaviors could define the respective roles of the three neurotransmitters.

Finally, although it is well beyond the scope of this review to describe exhaustively the regulation of the forebrain cholinergic systems, it must be mentioned that also other neurotransmitters contribute to this regulation. Dopaminergic neurons activate the forebrain cholinergic neurons [8, 10, 11]; serotonin modulates the cortical cholinergic system with various effects depending on the receptor subtype activated [9, 22, 25, 49]; noradrenaline exerts a tonic inhibitory regulation of frontal ACh release [47];

cholecystokinin stimulates cortical ACh release [31]; VIP increases hippocampal ACh release through a presynaptic mechanism [33]. This short and by no means complete list of neurotransmitters acting upon the forebrain cholinergic systems is enough to demonstrate the complexity of their regulation and raises the question of their respective involvement in different behavioral conditions. It also suggests that the forebrain cholinergic systems is the critical final pathway of a large number of inputs influencing the cognitive processes.

References

- [1] Acquas E., Wilson C., Fibiger H.C., Conditioned and unconditioned stimuli increase frontal cortical and hippocampal acetylcholine release, effects of novelty, habituation, and fear, *J. Neurosci.* 16 (1996) 3088–3096.
- [2] Acquas E., Wilson C., Fibiger H.C., Pharmacology of sensory stimulation-evoked increases in frontal cortical acetylcholine release, *Neuroscience* 85 (1998) 73–83.
- [3] Aloisi A.M., Casamenti F., Scali C., Pepeu G., Carli G., Effects of novelty, pain and stress on hippocampal extracellular acetylcholine levels in male rats, *Brain Res.* 748 (1997) 219–226.
- [4] Bartolini L., Casamenti F., Pepeu G., Aniracetam restores object recognition impaired by age, scopolamine, and nucleus basalis lesions, *Pharmacol. Biochem. Behav.* 53 (1995) 277–283.
- [5] Blaker W.D., Peruzzi G., Costa E., Behavioral and neurochemical differentiation of specific projections in the septal-hippocampal cholinergic pathway of the rat, *Proc. Natl. Acad. Sci. USA* 81 (1984) 1880–1882.
- [6] Blandina P., Giorgetti M., Bartolini L., Cecchi M., Timmerman H., Pepeu G., Giovannini M.G., Inhibition of cortical acetylcholine release and cognitive performance by histamine H3 receptor activation in rats, *Br. J. Pharmacol.* 119 (1996) 1656–1664.
- [7] Bonanno G., Ruelle A., Andrioli G.C., Raiteri M., Cholinergic nerve terminals of human cerebral cortex possess a GABA transporter whose activation induces release of acetylcholine, *Brain Res.* 539 (1991) 191–195.
- [8] Casamenti F., Deffenu G., Abbamondi A., Pepeu G., Changes in cortical acetylcholine output induced by modulation of the nucleus basalis, *Brain Res. Bull.* 16 (1986) 689–695.
- [9] Consolo S., Arnaboldi S., Ramponi S., Nannini L., Ladinsky H., Baldi G., Endogenous serotonin facilitates in vivo acetylcholine release in rat frontal cortex through 5-HT1B receptors, *J. Pharmacol. Exp. Ther.* 277 (1996) 823–830.
- [10] Day J., Fibiger H.C., Dopaminergic regulation of cortical acetylcholine release, *Synapse* 12 (1992) 281–286.
- [11] Day J.C., Fibiger H.C., Dopaminergic regulation of septo-hippocampal cholinergic neurons, *J. Neurochem.* 63 (1994) 2086–2092.
- [12] Day J., Damsma G., Fibiger H.C., Cholinergic activity in the rat hippocampus, cortex and striatum correlates with locomotor activity, an in vivo microdialysis study, *Pharmacol. Biochem. Behav.* 38 (1991) 723–729.
- [13] Decker M.W., McGaugh J.L., The role of interactions between the cholinergic system and other neuromodulatory systems in learning and memory, *Synapse* 7 (1991) 151–168.
- [14] Dutar P., Rascol O., Jobert A., Lamour Y., Modulation of the excitability of septohippocampal terminals in the rat, relation to neuronal discharge rate, *Brain Res.* 418 (1987) 98–110.
- [15] Ennaceur A., Meliani K., Effects of physostigmine and scopolamine on rat's performances in object-recognition and radial maze tests, *Psychopharmacology* 109 (1992) 321–330.
- [16] Everitt B.J., Robbins T.W., Central cholinergic systems and cognition, *Annu. Rev. Psychol.* 48 (1997) 649–684.
- [17] Giorgetti M., Bacciottini L., Giovannini M.G., Blandina P., GABAergic modulation of cortical acetylcholine release in vivo, *Abs. Soc. Neurosci.* 22 (1996) 1255.
- [18] Giorgetti M., Bacciottini L., Bianchi L., Giovannini M.G., Cecchi M., Blandina P., GABAergic mechanisms in histamine H3 receptor inhibition of K⁺-evoked release of acetylcholine from rat cortex in vivo, *Inflamm. Res.* 46 (1997) S33–S34.
- [19] Giovannini M.G., Mutolo D., Bianchi L., Michelassi A., Pepeu G., NMDA receptor antagonists decrease GABA outflow from the septum and increase acetylcholine outflow from the hippocampus, a microdialysis study, *J. Neurosci.* 14 (1994) 1358–1365.
- [20] Giovannini M.G., Giovannelli L., Bianchi L., Kalfin R., Pepeu G., Glutamatergic modulation of cortical acetylcholine release in the rat, a combined in vivo microdialysis, retrograde tracing and immunohistochemical study, *Eur. J. Neurosci.* 9 (1997) 1678–1689.
- [21] Giovannini M.G., Bartolini L., Kopf S.R., Pepeu G., Acetylcholine release from the frontal cortex during exploratory activity, *Brain Res.* 784 (1998) 218–227.
- [22] Giovannini M.G., Ceccarelli I., Molinari B., Cecchi M., Goldfarb J., Blandina P., Serotonergic modulation of acetylcholine from cortex of freely moving rats, *J. Pharmacol. Exp. Ther.* (1998), in press.
- [23] Giovannini M.G., Rakowska A., Della Corte L., Bianchi L., Pepeu G., Activation of non-NMDA receptors stimulates acetylcholine and GABA release from dorsal hippocampus, a microdialysis study in the rat, *Neurosci. Lett.* 243 (1998) 152–156.
- [24] Gorman L.K., Pang K., Frick K.M., Givens B., Olton D.S., Acetylcholine release in the hippocampus: effects of cholinergic and GABAergic compounds in the medial septal area, *Neurosci. Lett.* 166 (1994) 199–202.
- [25] Hirano H., Day J., Fibiger H.C., Serotonergic regulation of acetylcholine release in the rat frontal cortex, *J. Neurochem.* 65 (1995) 1139–1145.
- [26] Imperato A., Puglisi-Allegra S., Zocchi A., Angelucci A., Stress-induced enhancement of dopamine and acetylcholine release in limbic structures, role of corticosterone, *Eur. J. Pharmacol.* 165 (1989) 337–338.
- [27] Imperato A., Puglisi-Allegra S., Casolini P., Angelucci L., Changes in brain dopamine and acetylcholine release during and following stress are independent of the pituitary-adrenocortical axis, *Brain Res.* 538 (1991) 11–117.
- [28] Inglis F.M., Fibiger H.C., Increases in hippocampal and frontal cortical acetylcholine release associated with presentation of sensory stimuli, *Neuroscience* 66 (1995) 81–86.
- [29] Inglis F.M., Day J.C., Fibiger H.C., Enhanced acetylcholine release in hippocampus and cortex during the anticipation and consumption of a palatable meal, *Neuroscience* 62 (1994) 1049–1056.
- [30] Kiss J., Patel A.J., Baimbridge K.G., Freund T.F., Topographical localization of neurons containing parvalbumin and

- choline acetyltransferase in the medial septum-diagonal band region of the rat, *Neuroscience* 36 (1990) 61–72.
- [31] Magnani M., Mantovani P., Pepeu G., Effect of cholecystokinin octapeptide and ceruletide on release of acetylcholine from cerebral cortex of the rat in vivo, *Neuropharmacology* 11 (1984) 1305–1309.
- [32] Malthe-Sørensen D., Odden D., Walaas I., Selective destruction by kainic acid of neurons innervated by putative glutamatergic afferents in septum and nucleus of the diagonal band, *Brain Res.* 182 (1980) 461–465.
- [33] Masuo Y., Matsumoto Y., Tokito F., Tsuda M., Fujino M., Effects of vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) on the spontaneous release of acetylcholine from the rat hippocampus by brain microdialysis, *Brain Res.* 611 (1993) 207–215.
- [34] Mitsushima D., Mizuno T., Kimura F., Age-related changes in diurnal acetylcholine release in the prefrontal cortex of male rats as measured by microdialysis, *Neuroscience* 72 (1996) 429–434.
- [35] Moor E., Schirm E., Jacsó J., Westerink B.H.C., Effects of neostigmine and atropine on basal and handling-induced acetylcholine output from ventral hippocampus, *Neuroscience* 82 (1998) 819–825.
- [36] Moore H., Sarter M., Bruno J.P., Bidirectional modulation of cortical acetylcholine efflux by infusion of benzodiazepine receptor ligands into the basal forebrain, *Neurosci. Lett.* 189 (1995) 31–34.
- [37] Nilsson O.G., Björklund A., Behaviour-dependent changes in acetylcholine release in normal and graft-reinnervated hippocampus, evidence for host regulation of grafted cholinergic neurons, *Neuroscience* 49 (1992) 33–44.
- [38] Orsetti M., Casamenti F., Pepeu G., Enhanced acetylcholine release in the hippocampus and cortex during acquisition of an operant behavior, *Brain Res.* 724 (1996) 89–96.
- [39] Ragozzino M.E., Pal S.N., Unick K., Stefani M., Gold P.E., Modulation of hippocampal acetylcholine release and spontaneous alternation scores by intrahippocampal glucose injections, *J. Neurosci.* 18 (1998) 1595–1601.
- [40] Rasmusson D., Szerb J.C., Cortical acetylcholine release during operant behaviour in rabbits, *Life Sci.* 16 (1975) 683–690.
- [41] Rasmusson D.D., Clow K., Szerb J.C., Modification of neocortical acetylcholine release and electroencephalogram desynchronization due to brainstem stimulation by drugs applied to the basal forebrain, *Neuroscience* 60 (1994) 665–677.
- [42] Rasmusson D.D., Szerb J.C., Jordan J.L., Differential effects of α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid and N-methyl-D-aspartate receptor antagonists applied to the basal forebrain on cortical acetylcholine release and electroencephalogram desynchronization, *Neuroscience* 72 (1996) 419–427.
- [43] Richardson R.T., DeLong M.R., Responses of primate nucleus basalis neurons to water rewards and related stimuli, in: Steriade M., Biesold D., (Eds.), *Brain cholinergic system*, Oxford University Press, Oxford, 1990, pp. 282–293.
- [44] Schäfer M.K.H., Eiden L.E., Weihe E., Cholinergic neurons and terminal fields revealed by immunohistochemistry for the vesicular acetylcholine transporter. I. Central nervous system, *Neuroscience* 84 (1998) 331–359.
- [45] Sivilotti L., Nistri A., GABA receptor mechanisms in the central nervous system, *Progr. Neurobiol.* 36 (1990) 35–92.
- [46] Tajima T., Endo H., Suzuki Y., Ikari H., Gotoh M., Iguchi A., Immobilization stress-induced increase of hippocampal acetylcholine and of plasma epinephrine, norepinephrine and glucose in rats, *Brain Res.* 720 (1996) 155–158.
- [47] Tellez S., Colpaert F., Marien M., Acetylcholine release in the rat prefrontal cortex in vivo, modulation by α_2 -adrenoceptor agonists and antagonists, *J. Neurochem.* 68 (1997) 778–785.
- [48] Yamamuro Y., Hori K., Tanaka Y., Ywano H., Nomura M., Septo-hippocampal cholinergic system under the discrimination learning task in the rat, a microdialysis study with the dual-probe approach, *Brain Res.* 684 (1995) 1–7.
- [49] Zhelyazkova-Savova M., Giovannini M.G., Pepeu G., Increase of cortical acetylcholine release after systemic administration of chlorophenylpiperazine in the rat, an in vivo microdialysis study, *Neurosci. Lett.* 236 (1997) 151–154.
- [50] Zucker J., Calkins D., Zabawska J., Lai H., Horita A., Effects of intraseptal drug administration on pentobarbital induced narcosis and hippocampal choline uptake, *Pharmacol. Biochem. Behav.* 4 (1987) 433–436.
- [51] Mizuno T., Kimura F., Attenuated stress response of hippocampal acetylcholine release and adrenocortical secretion in aged rats, *Neurosci. Lett.* 222 (1997) 49–52.

Enzymes hydrolyzing organophosphates as potential catalytic scavengers against organophosphate poisoning

Patrick Masson^a, Denis Josse^{a, b}, Oksana Lockridge^b, Nathalie Viguié^a,
Claire Taupin^a, Cyril Buhler^a

^aCentre de Recherches du Service de Santé des Armées, Department of Toxicology, Enzymology Unit,
BP 87, 38702 La Tronche cedex, France

^bUniversity of Nebraska Medical Center, Eppley Institute, 600 South 42nd St., Omaha 68198-9805, NE, USA

Abstract — Enzymes hydrolyzing organophosphates could be used as catalytic scavengers for treatment of organophosphate poisoning and for decontamination. Two organophosphorus hydrolases (OPH) were selected: the *Flavobacterium sp./Pseudomonas diminuta* phosphotriesterase (PTE) and human paraoxonase (HuPON). Genes encoding these enzymes were cloned and functional recombinant enzymes expressed. PTE was expressed in *E. coli*. Natural HuPON was purified from human plasma; recombinant HuPON was expressed in human embryonic kidney 293 T cells. Although HuPON displays interesting catalytic properties, a site-directed mutagenesis program was undertaken to improve its catalytic efficiency. PTE has high efficiency in hydrolysis of organophosphates, including nerve agents. PTE injected in rat has a half-life of 100 min. However, to overcome pharmacokinetic problems of injected OPH and/or immunological incompatibility, the model enzyme (recombinant PTE) was immobilized onto a hollow-fiber reactor. This reactor designed for extracorporeal blood circulation is under experimentation for post-exposure detoxification. (©Elsevier, Paris)

Résumé — Enzymes hydrolysant les organophosphorés, épurateurs catalytiques potentiels contre l'intoxication par les organophosphorés. Les enzymes hydrolysant les organophosphorés (OPH) sont des épurateurs catalytiques potentiels qui pourraient être utilisés pour le traitement de l'intoxication organophosphorée, ainsi que pour la décontamination. Nous nous sommes intéressés à deux enzymes : la phosphotriesterase (PTE) de *Flavobacterium sp./Pseudomonas diminuta* et la paraoxonase humaine (HuPON). Les gènes codant pour ces enzymes ont été clonés et les enzymes recombinantes fonctionnelles exprimées. La PTE a été exprimée dans *E. coli*. La PON naturelle a été purifiée à partir du plasma humain ; l'HuPON recombinante a été produite dans des cellules embryonnaires de rein humain 293 T. Bien que HuPON possède des propriétés catalytiques intéressantes, nous avons entrepris d'augmenter son efficacité catalytique par mutagenèse dirigée. La PTE possède une activité catalytique élevée vis-à-vis de nombreux OPs, dont les neurotoxiques de guerre. La demi-vie biologique de cette enzyme injectée au rat par voie intraartérielle est de l'ordre de 100 min. Cependant, pour éviter les éventuels problèmes pharmacocinétiques et/ou immunologiques consécutifs à l'injection d'OPH, l'épuration du sang pourrait être réalisée dans un réacteur à OPH grâce à un système de circulation extracorporelle. Dans ce but, la PTE, enzyme modèle, a été immobilisée dans un réacteur à fibres creuses. (©Elsevier, Paris)

organophosphate poisoning / scavenger / organophosphorus hydrolase (OPH) / phosphotriesterase (PTE) / paraoxonase (PON)

1. Introduction

In the past 10 years, pretreatment and treatment of organophosphate poisoning have made progress. However, the persistence for long time after absorption of organophosphate molecules (OP) in depot sites from where they are slowly and continuously released into the blood can cause clinical complications as seen in patients severely poisoned by OP insecticides [29]. Indeed, from blood, OP rapidly reaches peripheral and central acetylcholinesterases that are maintained inhibited at a high level (*figure 1*). The presence of endogenous scavengers in plasma probably reduces the long-term toxicity of OPs. Thus, enzyme-catalyzed degradation or removal of OP in blood could greatly improve the efficiency of the classical treatment of organophosphate poisoning. In this connection, it should be remembered that hemodiafiltration was successfully applied in Tokyo to

a patient with severe sarin poisoning resistant to standard drug therapy [30].

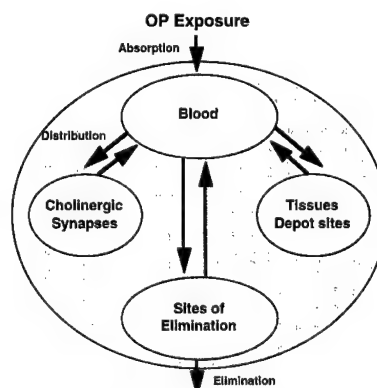


Figure 1. General scheme showing availability and metabolism of OP in human body.

Organophosphate-degrading enzymes and other catalytic and pseudocatalytic scavengers have gained in interest and the technology of biological scavengers is now emerging. Catalytic scavengers could be used as detoxifying drugs. Intravenous administration of catalytic scavengers is expected to reduce the risk of post-exposure OP poisoning due to the slow release in blood of OP from peripheral compartments. In the near future these catalysts could also be a part of the package of countermeasures for selective harmless decontamination of skin and eyes and for medical protection (prophylaxis) against nerve agents. Also, genetically engineered microorganisms could be used for OP detoxification, and environment/equipment decontamination [5].

2. OP-degrading enzymes

Numerous OP-degrading enzymes have been isolated from animal tissues and microorganisms. These enzymes are structurally different. *Alteromonas* prolidases (E.C. 3.4.13.9) are very active and have considerable potential for nerve agent decontamination [6]. Mutants of human butyrylcholinesterase (E.C. 3.1.1.8) capable of hydrolyzing OPs have been designed. These mutants hydrolyze various OPs, including the nerve agents soman, sarin and VX [18–20]; however, their catalytic activity is still too low to be of practical interest. To date, the most active organophosphate-degrading enzyme is the phosphotriesterase (E.C. 3.1.8.1; PTE) that has been isolated from *Pseudomonas diminuta* and *Flavobacterium* sp. (table I). This enzyme is a dimeric zinc metalloenzyme of 72 kDa; the 3-D structure of both apo- and holo enzyme has been solved [1]. Functional recombinant PTE expressed in *E. coli* administered intravenously was found to protect mice against toxic doses of soman, sarin or paraoxon [2, 25, 26]. Pretreatment by PTE and carbamates was found to be very effective against DFP [27] or sarin [28] poisoning. Human paraoxonase (E.C. 3.1.8.1; HuPON) is

a plasma 44 kDa hydrophobic monomeric glycoprotein associated with HDL apo A-I. Ca^{2+} is required for stability and catalytic activity. Two allelozymes (R191 and Q191) have been recognized; they exhibit differential activity toward OP substrates (table I). Little information is available on the active site characteristics of PON [23], its sequence shows no similarity with any other proteins, and its 3-D structure has not yet been resolved. Although PON has not yet been used in protection trials against chemical warfare agents, the relative resistance of rabbit to OP correlated with high endogenous PON activity in serum (10 times higher than in human serum), suggesting that PON has good detoxifying capacity [13]. Therefore, we focused our attention on two enzymes that look promising: the bacterial PTE and HuPON.

3. Rationale of *in vivo* enzyme-catalyzed hydrolysis of OPs

Even in the most severe poisoning, the concentration of OP in blood is thought to be well-below the K_m of OP-degrading enzymes. For example, the concentration of paraoxon in blood of patients poisoned by parathion has been estimated to range between 0 and 400 nM [9]. K_m values for paraoxon were found to be 271 μM for paraoxonase B [22], and 68 μM for Zn-wild type PTE [15]. Paraoxon concentrations in blood are at least 175 times lower than the lowest K_m , supporting the contention that OPH-catalyzed hydrolysis of paraoxon in blood is first order. Thus, OPs circulating in blood of poisoned patients are expected to be hydrolyzed by OPHs according to first order kinetics:

$$v = k_{\text{cat}}/K_m \cdot [E][\text{OP}]$$

where $[E]$ is the OPH active site concentration, $[\text{OP}]$ is the concentration of OP and $(k_{\text{cat}}/K_m) \cdot [E]$ is the first-order rate constant of hydrolysis. The amount

Table I. Bimolecular rate constant k_{cat}/K_m ($\text{M}^{-1} \text{min}^{-1}$) of two OP hydrolases.

OP	HuPON		<i>Pseudomonas diminuta</i> PTE
	A (Q191)	B (R191)	(Zn-wt)
Paraoxon	6.8×10^{5a}	2.4×10^{6a}	2.03×10^{9b}
DFP	3.7×10^{4c}	ND	5.8×10^{8c}
Sarin	9.1×10^{5d}	6.8×10^{4d}	4.8×10^{6f}
Soman	2.8×10^{6d}	2.1×10^{6d}	6.0×10^{5f}
VX	—	—	—

^apH 7.4 at 37 °C [22]; ^bpH 9.0 at 25 °C [15]; ^cpH 7.4, 1 mM CaCl_2 at 25 °C, Josse et al., unpublished; ^dpH 8.5, 1 M NaCl and 2 mM; ^epH 7.2 at 37 °C [16] CaCl_2 at 25 °C [7]; ^fpH 7.2 at 37 °C [8].

of enzyme that should be injected in the bloodstream to reduce the organophosphate concentration by 50% per unit time is:

$$[E] = \left\{ \frac{\ln 2}{k_{cat} / K_m} \right\} / t$$

Under steady state conditions, taking the volume of plasma as 40 mL per kg of body weight and the median time for a complete cycle of a molecule in the blood circulation as $t = 1$ min to decrease toxic plasma concentrations of paraoxon by half in 1 min, one should inject at least 75 μ g of *Pseudomonas diminuta* PTE or 40 mg of human paraoxonase. However, the concentration in blood of injected enzyme $[E]$ is decreasing with time due to the fact that enzyme is eliminated from the bloodstream. For example, the clearance of asialoglycoproteins is very fast because these proteins are trapped by hepatocyte receptors which recognize galactosyl residues. The kinetics of elimination can be expressed by the general equation:

$$[E] = \sum_i C_i e^{-k_i t}$$

where i is the number of compartments, coefficients C_i depend on the dose and k_i are related to the actual rate constant of elimination. For a one-compartment system, the biological half-time is $t_{1/2} = \ln 2/k$. All these parameters have to be taken into account to determine the dose to be administered and the interval times between repeated injections.

4. Phosphotriesterase

The gene of *Flavobacterium/Pseudomonas* PTE was cloned in pET17b and functional recombinant PTE was expressed in *E. coli* BL 21. The enzyme was purified to homogeneity by a three-step chromatography method (CM Sepharose, Green agarose and size exclusion on AcA54). Another enzyme was made in which the signal peptide was deleted and a 10 His tag was added at the N-terminus. This enzyme was expressed in *E. coli* HMS 174, and it was purified in a single step by metal-chelate affinity chromatography.

Pharmacokinetic study of recombinant PTE flash-injected in the femoral artery of rats showed a circulating half-life long enough ($t_{1/2} = 105 \pm 5$ min) to be of pharmacological interest [24]. However, engineering of human-compatible injectable PTE might be difficult to achieve. To circumvent this problem,

we considered the possibility for post-exposure detoxification by circulating blood through a cartridge containing immobilized PTE. Several enzymes have been used for extracorporeal therapy for years [14]. Such an approach in emergency medicine is not unrealistic since artero-venous hemofiltration on hollow-fiber cartridges has been used by rescuers to avoid crush-syndrome in earthquake casualties for instance. Although the implementation of such devices needs skilled medical staff, this would pose no problem at field hospital level. Our first results showed that storage stability of recombinant PTE was considerably increased by immobilization on a Sepharose 4B matrix, but K_m for paraoxon of immobilized enzyme was increased at least 15 times. This corroborates results obtained with immobilized PTE in other types of reactors [3, 4, 17]. Since hollow fiber based reactors have been proven to be safe and efficient, e.g., for extracorporeal circulation across artificial organs, removal of toxins from blood, cancer therapy, we chose to immobilize PTE onto hollow fibers. The reactor is a bundle of celulosic hollow fibers on which PTE was coupled by cyanogen bromide. Although preliminary results demonstrated the feasibility of this system, it must be optimized to be of medical interest.

5. Paraoxonase

We have undertaken both chemical and site-directed mutagenesis studies of PON to identify important active site residues, the ultimate goal of this work being to make PON mutants capable of degrading OPs with an efficiency similar to that of bacterial PTE. The natural enzyme was purified from human plasma by affinity chromatography on blue Cibacron gel according to [10]. The gene of HuPON was cloned in pGS vector and transiently expressed in 293T/17 cells. A 6 His tag was linked on the C-terminal position. Recombinant PON was purified by metal-chelate affinity chromatography. The His tag did not affect PON activity. Chemical modification of natural HuPON was performed to identify important amino acids. As for PTE, modification with diethylpyrocarbonate (DEPC) provided no clear evidence for the presence of histidine residues in the active site, but the pH dependence study of DEPC inactivation giving pK_a of 6.28 and 6.98 for modified residues, argues for the presence of histidine (H) residues. PON inactivation induced at $pH < 6$ suggested that carboxylic residues (D, E) may be involved in Ca^{2+} binding to the active site. Lastly, chemical modification by N-bromosuccinimide (NBS) clearly indicated that tryptophan (W) residues play a role in activity. In addition, the luminescence

spectrum of the calcium analog terbium supported the contention that Ca^{2+} interacts with a tryptophan residue [11]. Tryptophan residue (s) may be involved in binding of aromatic substrates.

Conserved residues W, H, D and E among known sequences of PON were selected for mutagenesis. Site-directed mutagenesis of the HuPON gene was

performed with PCR and Pfu polymerase. Mutagenesis was performed on the four tryptophan residues ($\text{W} \rightarrow \text{A/F}$), on the histidine residues ($\text{H} \rightarrow \text{N}$), on aspartate /glutamate residues ($\text{D/E} \rightarrow \text{A}$) and on asparagine residues ($\text{N} \rightarrow \text{A}$). Transient expression of mutated PON in human embryonic kidney 293 T/17 cells was used for screening catalytic activity of mu-

Table II. Kinetic parameters of selected HuPON mutants toward two OPs and phenylacetate.

<i>OP</i>	<i>Mutant</i>	<i>Relative K_m^a</i>	<i>Relative k_{cat}^a</i>
Chlorpyrifosoxon ^b	W193A/F	1.51/1.55	0.47/1.37
	W201A/F	1.08/1.18	0.34/2.11
	W253A/F	1.21/1.63	2.49/2.90
	W280A/F/L	ND/1.66/1.90	< 0.01/0.94/0.18
	H114N	ND	< 0.01
	H133N	ND	< 0.01
	H154N/Y	ND/1.10	< 0.01/0.52
	H160N	1.16	0.43
	H242N	ND	< 0.01
	H245N	1.02	0.28
	H250N	0.75	0.61
	H284N	ND	< 0.01
	H 347N	1.52	0.15
	D168A	ND	< 0.04
	D182A	ND	0.07
	D268A	ND	< 0.04
	D278A	2.23	< 0.04
	E194A	ND	< 0.04
Diazoxon ^c	W193A/F	1.38/1.10	0.39/0.88
	W201A/F	1.36/3.67	0.34/4.19
	W253A/F	0.93/1.64	1.99/3.60
	W280A/F/L	ND/0.81/1.25	< 0.01/0.56/0.06
	H114N	ND	< 0.01
	H133N	ND	< 0.01
	H154N/Y	ND/1.25	< 0.01/0.63
	H160N	1.37	0.44
	H242N	ND	< 0.01
	H245N	1.16	0.32
	H250N	0.71	0.58
	H284N	ND	< 0.01
	H347N	1.34	0.12
	D168A	ND	< 0.05
	D182A	ND	< 0.05
	D278A	ND	0.05
	E194A	3.32	< 0.05
	N132A	1.13	0.06
Phenylacetate ^d	W280L	0.59	0.15
	H154Y	0.93	0.48
	D53A	—	0
	D107A	ND	< 0.8
	D121A	ND	< 0.8
	D168	0.77	0.04
	D182A	1.56	0.04
	D268	ND	0.04
	D273A	ND	< 0.8
	D278A	1.14	0.08
	E32A	ND	< 0.8
	E48A	ND	< 0.8
	E52A	—	0
	E194A	1.57	0.12
	N132A	1.32	0.06

^a K_m and k_{cat} relative to those of wild-type enzyme taken as 1.

^{b, c, d}pH 8.5, 2 mM CaCl_2 , 1 M NaCl at 25 °C.

tants toward phenylacetate and two OPs: diazoxon and chlorpyrifos oxon (*table II*). Mutagenesis results showed that eight residues are important: W280, H114, H133, H154, H242, H284, E52 and D53. These residues very likely play a key role in the active site machinery, e.g., W280 as a component of the substrate binding site and H residues as nucleophile poles, but we cannot rule out the possibility that some of them are involved in the Ca^{2+} binding sites which control both the enzyme structural stability and activity. E52 and D53 are very likely involved in Ca^{2+} binding [12]. Six residues are essential for activity: N132, D168, D182, E194, D268 and D278.

6. Future prospects

Mutants of HuPON having improved OPH activity and enzymes having catalytic or pseudocatalytic OPH activity have to be designed; they can be reasonably expected in the very near future. For that purpose, more research has to be done on the catalytic mechanisms of OPH and quantitative structure-activity relationships. In a very near future, resolution of the X-ray structure of rabbit and human PON should also stimulate research in the field. Lessons from nature will help to design mutants of cholinesterases and carboxylesterases having high OPH activity. It is, for instance, noteworthy that insecticide resistance of a blowfly is due to a single amino acid mutation $\text{G} \rightarrow \text{D}$, in a position homologous to G117 in human butyrylcholinesterase, that converts a carboxylesterase to an OPH [2].

Controlling glycan heterogeneity of sialoglycoproteins by optimization of their biosynthesis in mammalian cells and carbohydrate engineering should slow down the clearance of therapeutic recombinant enzymes (e.g., HuPON) from blood circulation. In the case of recombinant bacterial enzymes, surface modifications (e.g., PEGylation) are expected to reduce immunogenicity. This should be an area of intensive effort in the next years. Moreover, new approaches such as the extracorporeal approach should be considered with particular interest.

Stability problems are also of major importance. Stable thermozymes for decontamination would have the advantage of storage at ambient temperature without loss of activity and to be usable under field conditions. Screening of bacteria that thrive in extreme environments (e.g., submarine hydrothermal vents, hot springs, very salty media) is underway. Some archaeobacterial strains have already been found to be capable of degrading a variety of organophosphates, including chemical warfare nerve agents.

Acknowledgments

Supported by the Service de Santé des Armées (France) and by grants from the Direction des Systèmes de Forces et de la Prospective (DRET No 96/02 and 97/08).

References

- [1] Benning M.M., Kuo J.M., Raushel F.M., Holden H.M., Three-dimensional structure of the bimolecular metal center of phosphotriesterase, *Biochemistry* 34, (1995) 7973–7978.
- [2] Broomfield C.A., A purified recombinant organophosphorus acid anhydrase protects mice against soman, *Pharmacol. Toxicol.* 70 (1992) 65–66.
- [3] Cadwell S.R., Raushel F.M., Detoxification of organophosphate pesticides using an immobilized phosphotriesterase from *Pseudomonas diminuta*, *Biotechnol. Bioeng.* 37 (1991) 103–109.
- [4] Cadwell S.R., Raushel F.M., Detoxification of organophosphate pesticides using a nylon based immobilized phosphotriesterase from *Pseudomonas diminuta*, *Appl. Biochem. Biotechnol.* 31 (1991) 59–73.
- [5] Chen W., Mulchandani A., The use of biocatalysts for pesticide detoxification, *TIBTECH* 16 (1998) 71–76.
- [6] Cheng T.-C., Liu L., Wang B., DeFrank J.J., Anderson D.M., Rastogi V.K., Hamilton A.B., Nucleotide sequence of a gene encoding an organophosphorus nerve agent degrading enzyme from *Alteromonas haloplanktis*, *J. Indust. Microb. Biotechnol.* 18 (1997) 49–55.
- [7] Davis H.G., Richter R.J., Keifer M., Broomfield C.A., Sowa J., Furlong C.E., The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin, *Nature Genet.* 14 (1996) 334–336.
- [8] Dumas D.P., Durst H.D., Landis W.G., Raushel F.M., Wild J.R., Inactivation of organophosphorus nerve agents by the phosphotriesterase from *Pseudomonas diminuta*, *Arch. Biochem. Biophys.* 277 (1990) 155–159.
- [9] Eyer F., Eyer P., in: 2nd Chem. Med. Def. Conf., Munich, Germany, 23–24 April, 1997.
- [10] Gan K.N., Smolen A., Eckerson H.W., La Du B.N., Purification of human serum paraoxonase/arylesterase, *Drug. Metab. Dispos.* 19 (1991) 100–106.
- [11] Josse D., Xie W.-H., Masson P., Shopfer L., Lockridge O., Tryptophan residue(s) as major components of the human serum paraoxonase active site, *Chem. Biol. Interact.* (1998), in press.
- [12] Josse D., Xie W.-H., Masson P., Lockridge O., Human serum paraoxonase (PON1): identification of essential amino acid residues by group-selective labelling and site-directed mutagenesis, *Chem. Biol. Interact.* (1998), in press.
- [13] Kaliste-Korhonen E., Tuovinen K., and Hänninen O., *Hum. Exp. Toxicol.* 15 (1996) 972–978.
- [14] Klein M.D., Langer R., Immobilized enzymes in clinical medicine: an emerging approach to new drug therapies, *TIBTECH* 4 (1986) 179–185.
- [15] Kuo J.N., Chae M.Y., Raushel F.M., Perturbations to the active site of phosphotriesterase, *Biochemistry* 36 (1997) 1982–1988.
- [16] Lai K., Stolowich N.J., Wild J.R., Characterization of P-S bond hydrolysis in organophosphorothioate pesticides by organophosphorus hydrolase, *Arch. Biochem. Biophys.* 318 (1995) 59–64.

- [17] LeJeune K.E., Mesiano A.J., Bower S.B., Grimsley J.K., Wild J.R., Russell A.J., Dramatically stabilized phosphotriesterase-polymers for nerve agent degradation, *Biotechnol. Bioeng.* 54 (1997) 105–114.
- [18] Lockridge O., Blong R.M., Masson P., Millard C.B., Broomfield C.A., A single amino acid substitution Gly117His, confers phosphotriesterase activity on human butyrylcholinesterase, *Biochemistry* 36 (1997) 786–795.
- [19] Millard C.B., Lockridge O., Broomfield C.A., Design and expression of organophosphorus acid anhydride hydrolase activity in human butyrylcholinesterase, *Biochemistry* 34 (1995) 15925–15933.
- [20] Millard C.B., Lockridge O., Broomfield C.A., Organophosphorus acid anhydride hydrolase activity in human butyrylcholinesterase: synergy results in a somanase, *Biochemistry* 37 (1998) 237–247.
- [21] Newcomb R.D., Campbell P.M., Ollis D.L., Cheah E., Russell R.J., Oakesholt J.G., A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly, *Proc. Natl. Acad. Sci. USA* 94 (1997) 7464–7468.
- [22] Smolen A., Eckerson H.W., Gan K.N., Hailat N., La Du B.N., Characteristics of the genetically determined allozymic forms of human serum paraoxonase/arylesterase, *Drug Metab. Dispos.* 19 (1991) 107–112.
- [23] Sorenson R.C., Primo-Parmo S.L., Kuo C.-L., Adkins S., Lockridge O., La Du B.N., Reconsideration of the catalytic center and mechanism of mammalian paraoxonase/arylesterase, *Proc. Natl. Acad. Sci. USA* 92 (1995) 7187–7191.
- [24] Taupin C., Rochu D., Lallement G., Masson P., Use of recombinant phosphotriesterase as an OP scavenger, Nieminen K., Raakkonen E. (Eds.), *Proc. Symposium on NBC Defence'97* (Häyinkää, Finland, 10–12 June, 1997), Finland, Finland Defense Forces Research Centre, 1997, pp. 180.
- [25] Tuovinen K., Kaliste-Korhonen E., Raushel F.M., Hänninen O., Phosphotriesterase - A promising candidate for use in detoxification of organophosphates, *Fundam. Appl. Toxicol.* 23 (1994) 578–584.
- [26] Tuovinen K., Kaliste-Korhonen E., Raushel F.M., Hänninen O., Protection of organophosphate-inactivated esterases with phosphotriesterase, *Fundam. Appl. Toxicol.* 31 (1996) 210–217.
- [27] Tuovinen K., Kaliste-Korhonen E., Raushel F.M., Hänninen O., Eptastigmine-phosphotriesterase combination in DFP intoxication, *Toxicol. Appl. Pharmacol.* 140 (1996) 364–369.
- [28] Tuovinen K., Kaliste-Korhonen E., Hänninen O., Comparison of phosphotriesterase and carbamates in acute sarin intoxication, Nieminen K., Raakkonen E. (Eds.), *Proc. Symposium on NBC Defence'97* (Häyinkää, Finland, 10–12 June, 1997), Finland, Finland Defense Forces Research Centre, 1997, pp. 156–160.
- [29] Willems J.L., De Bisschop H.C., Cholinesterase reactivation in organophosphorus poisoned patients depends on the plasma concentrations of the oxime pralidoxime methylsulphate and the organophosphate, *Arch. Toxicol.* 67 (1993) 79–84.
- [30] Yokoyama K., Blood purification for severe sarin poisoning after the Tokyo subway attack, *JAMA* 274 (1995) 379.

Four acetylcholinesterase genes in the nematode *Caenorhabditis elegans*

Martine Arpagaus^{a,b}, Didier Combes^{a,b}, Emmanuel Culetto^{a,b}, Marta Grauso^{a,c},
Yann Fedon^a, Rita Romani^{a,c}, Jean-Pierre Toutant^a

^aGroupe Cholinestérases, DCC-Inra, 2, place Viala, 34060 Montpellier cedex 1, France

^bBiologie des Invertébrés, Inra, boulevard Meilland, 06606 Antibes, France

^cDepartment of Experimental Medicine, University of Perugia, Via del Giochetto, 06100 Perugia, Italy

Abstract — Whereas a single gene encodes acetylcholinesterase (AChE) in vertebrates and most insect species, four distinct genes have been cloned and characterized in the nematode *Caenorhabditis elegans*. We found that *ace-1* (mapped to chromosome X) is prominently expressed in muscle cells whereas *ace-2* (located on chromosome I) is mainly expressed in neurons. *Ace-x* and *ace-y* genes are located in close proximity on chromosome II where they are separated by only a few hundred base pairs. The role of these two genes is still unknown. (©Elsevier, Paris)

Résumé — Quatre gènes d'acétylcholinestérase chez le nématode *Caenorhabditis elegans*. À l'inverse de la situation des vertébrés et de la majorité des insectes, chez qui un gène unique code pour l'acétylcholinestérase (AChE), quatre gènes d'AChE ont été clonés et caractérisés chez *Caenorhabditis elegans*. Le gène *ace-1* (localisé sur le chromosome X) et le gène *ace-2* (chromosome I) assurent respectivement l'expression de l'AChE dans les tissus musculaire (*ace-1*) et nerveux (*ace-2*). Les gènes *ace-x* et *ace-y* ne sont séparés que de quelques centaines de paires de bases sur le chromosome II et leur rôle est pour l'instant inconnu. (©Elsevier, Paris)

acetylcholinesterases / Ace genes / *Caenorhabditis elegans* / nematodes

1. Introduction

Plant parasitic nematodes are devastating pathogens, responsible for an estimated annual \$100 billion loss in crops worldwide [5]. Chemical nematicide treatments include carbamates and organophosphates that inhibit acetylcholinesterase (AChE) and lead to inhibition of the neuromuscular transmission and death. *Caenorhabditis elegans* is a free living nematode which constitutes however an useful model of parasitic species [12]. Whereas a single gene encodes AChE in vertebrates [20] and in the majority of insects [11], the situation is more complex in *Caenorhabditis elegans*. Initial studies clearly identified two kinetically distinct major classes of AChE, A and B, encoded by two genes: *ace-1*, located on chromosome X, and *ace-2*, located on chromosome I [7, 14, 15]. Later, a third class of AChE (class C, accounting for only 5% of total AChE activity) was characterized by its very low K_m for ACh and its high resistance to eserine. Class C AChE was shown to be encoded by *ace-3*, a locus on chromosome II [16]. Finally, a careful examination of class C mutants suggested the existence of a fourth class (D) of AChE that represented less than 0.1% of the total AChE activity [21]. Since no mutation in class D AChE was isolated, the corresponding *ace-4* locus was neither identified nor chromosome-mapped.

The complete coding sequences of *ace-1* in *C. elegans* [2] and in the related nematode species *C.*

briggsae [9] have been reported. We now present additional data on the sequence of all *ace* genes [10], the tissue-specific expression of *ace-1* and *ace-2*, and on the peculiar genomic organization of *ace-x* and *ace-y*, two *ace* genes located in close association on chromosome II. Because of the close proximity of these two genes, we cannot yet identify which of these two genes encodes the class C AChE (and should be named *ace-3*), the other being *ace-4*.

1.1. *Ace-1*

Ace-1 was initially cloned by PCR with degenerate oligonucleotides deduced from the sequences EDCLYLN and FGESAG that are conserved in the whole cholinesterase family. The deduced amino acid sequence possessed all characteristic features of an acetylcholinesterase. The cDNA was expressed in Sf9 cells transfected by a recombinant baculovirus: the secreted enzyme had catalytic properties compatible with those of class A AChE [2]. Finally, using the strain p1000 of *C. elegans* that presents a dramatic reduction of class A AChE, we found a single nucleotide change in *ace-1* introducing a stop codon TGA in place of the codon TGG encoding W(84) ([24], conventional numbering of amino acids [19]). We have compared the sequences of *ace-1* in *C. elegans* and in the closely-related species *C. briggsae*. The coding sequence of *ace-1* in the two species is highly conserved, but intronic sequences and

3'UTR are largely divergent (Culetto et al., submitted). We then cloned a 2.4 kb fragment of the 5'UTR of *ace-1* in the two *Caenorhabditis* species, and found that transfection of *ace-1* gene including this upstream region was sufficient to restore a normal movement to the uncoordinated double mutant *ace-1/ace-2* of *C. elegans*, and a normal distribution of histochemically-detectable AChE activity (Culetto et al., submitted). In order to study the tissue-specific expression of *ace-1* during development and in the adult, we transfected translational fusions of 2.4 kb of *ace-1* 5'UTR and the reporter gene of green fluorescent protein (GFP). This construct is expressed in all body-wall muscle cells, in the three pharyngeal pm5 muscle cells, and in a few neurons of the head, tail and retrovesicular ganglia (Culetto et al., submitted). A comparison of conserved sequences in the 5'UTR in *C. elegans* and *C. briggsae*, and GFP expression driven by different deletions of this region, shows that a distal conserved block is responsible for the expression of *ace-1* in body-wall muscle cells, and that another block is responsible for the expression in pharyngeal muscle cells (Culetto et al., submitted).

1.2. *Ace-2*

No additional *ace* sequences were obtained by PCR using the initial sense primer EDCLYLN used for *ace-1*. We then used RT-PCR and degenerate primers deduced from the sequence GSEMW(84)N (*Torpedo*) and GTEMW(84)N (mammalian AChEs) in combination with the reverse primer FGESAG. Total mRNAs from the strain p1000 (null mutation in *ace-1*) were used, in order to reduce the annealing of the primers to *ace-1* mRNAs [24]. Two different clones (40 and 48) with the expected size (370 nt) were isolated that presented 52% and 53% identity with *ace-1* at the amino acid level (55% identity between clones 40 and 48). Probes from these two clones were successively hybridized on a YAC grid. This indicated that clone 40 hybridized with two YACs on chromosome I (Y44E3 and Y52G11), in a region compatible with the previous genetic mapping of *ace-2* [7]. The deduced amino acid sequence showed all characteristic features of an acetylcholinesterase (see legend to figure 1). A 2.5 kb fragment of the 5' flanking region of *ace-2* was cloned in *C. elegans* and used in translational fusions with GFP. Transfection of these constructs led to stable transformants, in which GFP was expressed in a large number of neurons but not in body-wall nor in pharyngeal muscles. Thus *ace-1* and *ace-2* have a very different pattern of tissue expression.

1.3. *Ace-x* and *ace-y*

Clone 48 isolated above hybridized to YACs Y48B6 and Y59C8 of chromosome II near the locus *unc-52*, a position expected for *ace-3*. BLAST analysis of *C. elegans* sequences data base revealed no identity with clone 48, indicating that this region of the *C. elegans* genome had not been sequenced yet. However, we found two sequences homologous to clone 48 in the data base of *C. briggsae*. These two sequences were located in a single phosmid of *C. briggsae* DNA (i.e., in close location on genomic DNA). One of these sequence presented 94% identity with clone 48, and was clearly its homologue in *C. briggsae*. The other sequence, which was neither *ace-1* nor *ace-2*, and presented 74% identity with clone 48, was thus a new *ace* sequence. We amplified its homologue in *C. elegans* using RT-PCR, and showed that it hybridized to the same two YACs Y48B6 and Y59C8 as clone 48. We thus have two *ace* genes, located in very close proximity on chromosome II, in *C. elegans* and *C. briggsae*. One of these two sequences corresponds to *ace-3*, the gene encoding class C AChE but it is not possible, at the moment, to decide which sequence is *ace-3* and which one corresponds to a new *ace* gene (*ace-4*). These sequences are thus called *ace-x* and *ace-y*, *ace-x* being located upstream of *ace-y*. RT-PCR indicated that the two genes *ace-x* and *ace-y* are transcribed in both species of *Caenorhabditis* [10]. Genomic sequencing in *C. elegans* showed that there are only 356 nt separating the stop codon of *ace-x* and the initiator ATG of *ace-y* and only 200 nt between the polyadenylation signal of *ace-x* and the trans-splicing site of *ace-y*. Figure 2 shows the genomic organization of *ace-x* and *ace-y* in *C. elegans*. The alignment of the four *ace* sequences from the initiator Met to the residue G202 is shown in figure 1 [10].

2. Conclusions and perspectives

2.1. *Ace-1* and *ace-2*

The apparent lack of effect of a single null mutation in either *ace-1* or *ace-2*, and the uncoordinated movements of the double null mutant *ace-1/ace-2* led to the conclusion that the two enzymes (class A and class B) are largely redundant, at least for the control of locomotion [7, 15]. In addition, the histochemical localizations of both enzymes are very similar [7]. Thus, the distinct pattern of tissue expression of the two genes, revealed with the reporter gene GFP, was unexpected. A simple explanation for the pattern of histochemical staining could be that

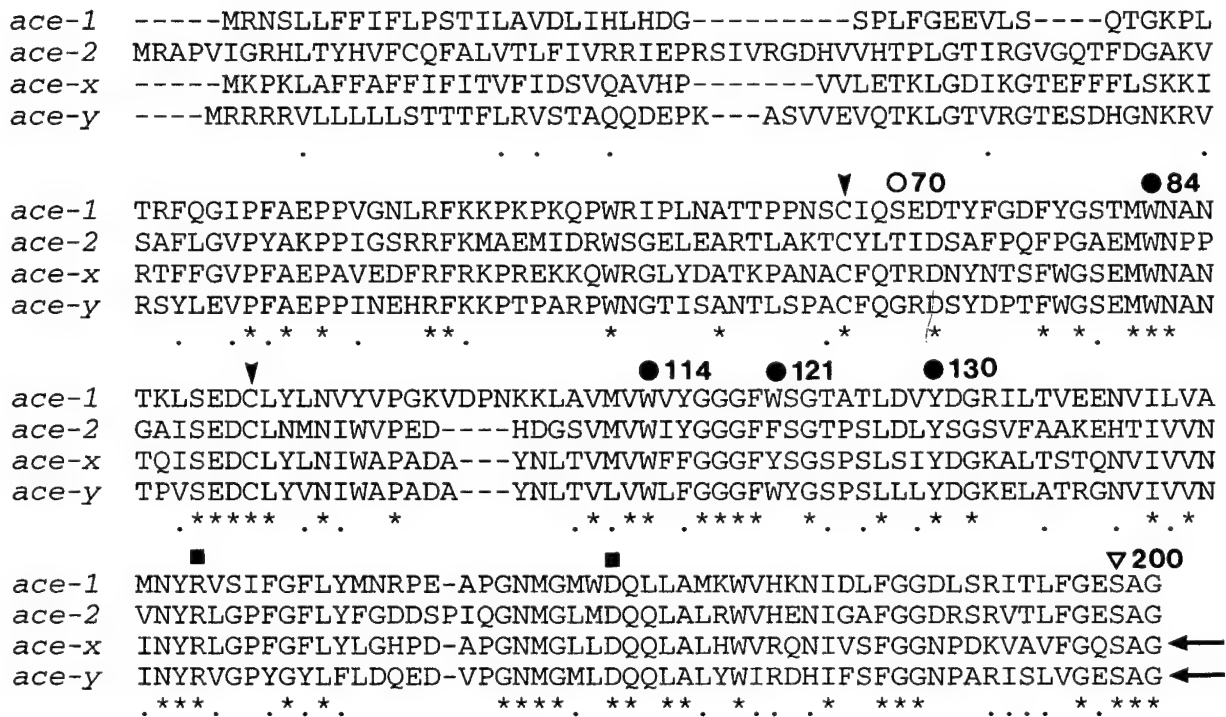


Figure 1. Alignment of the N-terminal amino acid sequences deduced from *ace* genes in *C. elegans*: *ace-1*, *ace-2*, *ace-x* and *ace-y*. The sequences extend from the initiator Met to G202. The numbering of amino acids is that of *Torpedo* AChE [19] where residue 1 is the mature N-terminus. In the region shown, the first S-S bond between C67 and C94 (arrowheads), W84 (the choline-binding site) and the active S200 are conserved, as well as R149 and D172 (squares) involved in a salt bridge. Out of the five aromatic residues lining the catalytic gorge in this region of *Torpedo* AChE, only four are conserved or semi-conserved (84, 114, 121, 130, filled circles). The Y70 residue of *Torpedo* AChE is replaced by a non-aromatic residue in all *ace* genes (open circle), as in *Drosophila* AChE. Horizontal arrows show the motif FGQSAG in *ace-x* and VGESAG in *ace-y*.

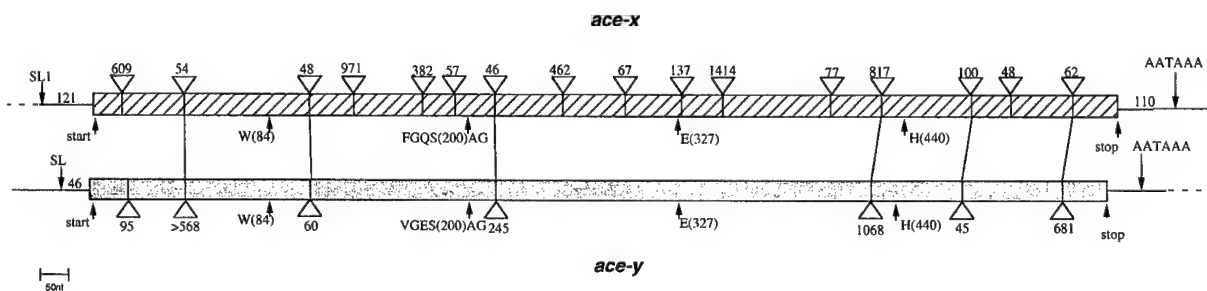


Figure 2. Genomic organization of *ace-x* and *ace-y* on chromosome II in *C. elegans*. *Ace-x* has 16 introns within the coding sequence against only 7 in *ace-y*. Six introns are in conserved position. A short insertion (8 amino acids) is present between introns 12 and 13 in *ace-x*. Note the short distance between the two contiguous genes: 356 nt between the stop codon of *ace-x* and the initiator ATG (start) of *ace-y*; 200 nt between the polyadenylation signal (AATAAA) and the site of trans-splicing (SL).

AChE is transported and preferentially accumulated at synapses, whether it is produced by muscle (class A) or nerve cells (class B).

2.2. *Ace-x* and *ace-y*

Another important result of our recent work is the identification of two additional *ace* genes (*ace-x* and *ace-y*), located close to each other on chromosome II, in both species of *Caenorhabditis*. The proximity of the two genes precludes, for the moment, the identification of *ace-x* or *ace-y* to *ace-3*, the only *ace* gene of chromosome II which has previously been defined in the literature. To allow a formal identification, sequencing of *ace-x* and *ace-y* in the known mutant strains of *ace-3* in *C. elegans* (alleles dc2, dc3, p1304, p1306 [7]) is currently under way. In vitro expression of *ace-x* and *ace-y* is also being performed for comparison of catalytic properties with those of class C [17]. The close arrangement of *ace-x* and *ace-y* in *C. elegans* genome is reminiscent of the organization of a number of polycistronic units (or operons) that have been described in this species [3]. In those units, a single promoter drives the coordinated transcription of several genes [3]. Whether this is the case for *ace-x* and *ace-y* is currently being studied.

2.3. Features of *ace* genes in *Caenorhabditis*

All *ace* sequences shared the following residues in conserved positions (numbering of *Torpedo* AChE, [19]): W84 as the choline-binding site; S200, E327 and H440 as the catalytic triad; three intrachain S-S bonds between C67-94, C254-265 and C402-521. Twelve (*ace-1*), eleven (*ace-2*) or thirteen (*ace-x* and *ace-y*) out of the fourteen aromatic residues lining the gorge in *Torpedo* AChE [2, 22] were conserved or semi-conserved. Interestingly, at the position of F288 in *Torpedo* AChE (a critical residue controlling the size of the acyl pocket and the substrate specificity of the enzyme), we found G in ACE-1 and M in ACE-2, whereas F288 was conserved in *ace-x* and *ace-y*. Both ACE-1 and ACE-2 enzymes hydrolyze significant amounts of butyrylthiocholine (see [14, 26]), and it will be interesting to test the substrate preference of the products of *ace-x* and *ace-y*. Also, the FGE(199)SAG motif present in *ace-1* and *ace-2*, as well as in all cholinesterases sequenced so far, is changed to FGQSAG in *ace-x* and VGESAG in *ace-y*. The directed mutation E199Q has been performed in vertebrate AChE (reviewed in [25]) and BChE [18]. In all cases, E199Q changes the inhibitor specificity, reduces the catalytic efficiency and the aging of the phosphorylated enzyme. Since *ace-3* is a major component of AChE in some phytoparasitic ne-

matodes such as *Meloidogyne* [6], this possible resistance to aging could be important for managing the use of organophosphates against nematodes in agriculture.

The active serine of the four *Caenorhabditis ace* genes is encoded by the codon TCn as in *Drosophila* AChE, whereas it is encoded by AGy in all vertebrate and prochordate AChEs [4, 23]. This supports Brenner's hypothesis on the evolution of AChEs, that predicts the existence of two lines of descent that could have originated separately [4].

Class A AChE (encoded by *ace-1*) possesses a typical hydrophilic C-terminus, with regularly spaced aromatic residues, as in the T subunits of vertebrate AChE or BChE [9, 13, 20]. Such a C-terminal fragment might adopt the conformation of an amphiphilic alpha helix crucial for oligomerization [8, 20]. In contrast, *ace-2*, *ace-x* and *ace-y* encode hydrophobic C-termini, which however show no clear homology with those of vertebrate H subunits or *Drosophila* AChE. In vitro expression is needed to decide whether these enzymes possess a glycolipid anchor as it was shown for AChE of class B in the nematode *Steinernema* [1]. Finally a free cysteine residue is lacking in the C-terminal region of ACE-X and ACE-Y. Recent observations have shown that a C-terminal free cysteine is not a prerequisite for dimerization of AChE. Thus in vitro expression of the *ace-x* and *ace-y* cDNAs constitutes a valuable model to test the relative contribution of the four helix bundle (the only source of non-covalent interactions between subunits) and the interchain disulfide bond in the formation of dimers.

Acknowledgments

This work was supported by grants from the Institut National de la Recherche Agronomique (M.A., J.P.T., Y.F.), the Ministère de la Recherche et de l'Enseignement Supérieur (E.C., D.C.), the Association Française contre les Myopathies (E.C., J.P.T., M.A.), the European Molecular Biology Organization (M.G., R.R.) and the Franco-Italian Galileo project (R.R., J.P.T.).

References

- [1] Arpagaus M., Richier P., Bergé J.-B., Toutant J.-P., Acetylcholinesterase of the nematode *Steinernema carpocapsae*, Eur. J. Biochem. 207 (1992) 1101-1108.
- [2] Arpagaus M., Fedon Y., Cousin X., Chatonnet A., Bergé J.-B., Fournier D., Toutant J.-P., cDNA sequence, gene structure, and in vitro expression of *ace-1*, the gene encoding acetylcholinesterase of class A in the nematode *Caenorhabditis elegans*, J. Biol. Chem. 269 (1994) 9957-9965.
- [3] Blumenthal T., Steward K., RNA processing and gene structure, in: Riddle D.L., Blumenthal T., Meyer B., Priess, J.R.

- (Eds.), *C. elegans* II, Cold Spring Harbor Laboratory Press, 1997, pp. 117–145.
- [4] Brenner S., The molecular evolution of genes and proteins: a tale of two serines, *Nature* 334 (1988) 528–530.
 - [5] Cai D., Kleine M., Kifle S., Harloff H.J., Sandal N.N., Marcker K.A., Klein-Lankhorst R.M., Salentijn E.M.J., Lange W., Stiekema W.J., Wyss U., Grundler F.M.W., Jung C., Positional cloning of a gene for nematode resistance in sugar beet, *Science* 275 (1997) 832–834.
 - [6] Chang S., Opperman C.H., Characterization of acetylcholinesterase molecular forms of the root-knot nematode, *Meloidogyne*, *Mol. Biochem. Parasitol.* 49 (1991) 205–214.
 - [7] Culotti J.G., Von Ehrenstein G., Culotti M.R., Russell, R.L., A second class of acetylcholinesterase-deficient mutants of the nematode *Caenorhabditis elegans*, *Genetics* 97 (1981) 281–305.
 - [8] Giles K., Interactions underlying subunit association in cholinesterases, *Prot. Engin.* 10 (1997) 677–685.
 - [9] Grauso M., Culetto E., Bergé J.-B., Toutant J.-P., Arpagaus, M., Sequence comparison of *ace-1*, the gene encoding acetylcholinesterase of class A, in the two nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*, *DNA Sequence* 6 (1996) 217–227.
 - [10] Grauso M., Culetto E., Fedon Y., Combes D., Toutant J.-P., Arpagaus, M., Existence of four acetylcholinesterase genes in the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*, *FEBS Lett.* 424 (1998) 279–284.
 - [11] Hall L.M.C., Spierer, P., The *Ace* locus of *Drosophila melanogaster*: structural gene for acetylcholinesterase with an unusual 5' leader, *EMBO J.* 5 (1986) 2949–2954.
 - [12] Howells R.E., Johnstone I., *Caenorhabditis elegans*: a model for parasitic nematodes, *Parasitol. Today* 7 (1991) 224–226.
 - [13] Jbilo O., L'Hermite Y., Talses V., Toutant J.-P., Chatonnet A., Acetylcholinesterase and butyrylcholinesterase expression in adult rabbit tissues and during development, *Eur. J. Biochem.* 225 (1994) 115–124.
 - [14] Johnson C.D., Russell, R.L., Multiple molecular forms of acetylcholinesterase in the nematode *Caenorhabditis elegans*, *J. Neurochem.* 41 (1983) 30–46.
 - [15] Johnson C.D., Duckett J.G., Culotti J.G., Herman R.K., Mcneely P.M., Russell R.L., An acetylcholinesterase-deficient mutant of the nematode *Caenorhabditis elegans*, *Genetics* 97 (1981) 261–279.
 - [16] Johnson C.D., Rand J.R., Herman R.K., Stern B.D., Russell R.L., The acetylcholinesterase genes of *C. elegans*: identification of a third gene (*ace-3*) and mosaic mapping of a synthetic lethal phenotype, *Neuron* 1 (1988) 165–173.
 - [17] Kolson D.L., Russell R.L., A novel class of acetylcholinesterase, revealed by mutations, in the nematode *Caenorhabditis elegans*, *J. Neurogenet.* 2 (1985) 93–110.
 - [18] Masson P., Fortier P.-L., Albaret C., Froment M.-T., Bartels C.F., Lockridge O., Aging of di-isopropyl-phosphorylated human butyrylcholinesterase, *Biochem. J.* 327 (1997) 601–607.
 - [19] Massoulié J., Sussman J.L., Doctor B.P., Soreq H., Velan B., Cygler M., Rotundo R., Shafferman A., Silman I., Taylor, P., Recommendations for nomenclature in cholinesterases, in: Shafferman A., Velan B. (Eds.), *Multidisciplinary Approaches to Cholinesterase Functions*, Plenum Press, New York, 1992, pp. 285–288.
 - [20] Massoulié J., Pezzementi L., Bon S., Krejci E., Vallette F.-M., Molecular and cellular biology of cholinesterases, *Prog. Neurobiol.* 41 (1993) 31–91.
 - [21] Stern B.D., Acetylcholinesterase from *Caenorhabditis elegans*: partial purification and immunochemistry of class C, and discovery of class D, Ph.D. Thesis dissertation (1986) University of Pittsburgh, PA, USA.
 - [22] Sussman J.L., Harel M., Frolow F., Offner C., Goldman A., Tokar L., Silman I., Atomic structure of acetylcholinesterase from *Torpedo californica*, *Science* 253 (1991) 872–879.
 - [23] Sutherland D., McClellan J.C., Milner D., Soong W., Axon N., Sanders M., Hester A., Kao Y.H., Poczek T., Routt S., Pezzementi, L., Two cholinesterase activities and genes are present in *Amphioxus*, *J. Exp. Zool.* 277 (1997) 213–229.
 - [24] Talses V., Culetto E., Schirru N., Bernardi H., Fedon Y., Toutant J.-P., Arpagaus M., Characterization of a null mutation in *ace-1*, the gene encoding class A acetylcholinesterase in the nematode *Caenorhabditis elegans*, *FEBS Lett.* 357 (1995) 265–268.
 - [25] Taylor P., Radic, Z., The cholinesterases: from genes to proteins, *Annu. Rev. Pharmacol. Toxicol.* 34 (1994) 281–320.
 - [26] Vellom D.C., Radic Z., Li Y., Pickering N.A., Camp S., Taylor, P., Amino acid residues controlling acetylcholinesterase and butyrylcholinesterase specificity, *Biochemistry* 32 (1993) 12–17.

Medical management of organophosphate-induced seizures

Guy Lallement^a, Frédéric Dorandeu^a, Pierre Filliat^a, Pierre Carpentier^a,
Valérie Baille^a, Guy Blanchet^b

^aCRSSA, Unité de Neuropharmacologie, BP 87, 38702 La Tronche cedex, France

^bDCSSA, AST/Bureau Recherches, cours des Maréchaux, 75012 Paris, France

Abstract — Recent studies concerning management of soman-induced seizures are reviewed. While drugs classically used against epilepsy in hospital appear ineffective against soman, muscarinic receptor blockers are shown to be able to prevent or stop seizures within the first 5 min after their onset. Benzodiazepine could also be considered as an emergency treatment useful during the first 10 min of seizure. Comparatively NMDA antagonists appear to be able to terminate soman-induced seizures even if the treatment is delayed after 40 min of epileptic activity. Drugs with both antimuscarinic and anti-NMDA properties may represent the most adequate pharmacological treatment to treat soman intoxication. However, the results obtained until now with these drugs must be completed in relation with their possible efficacy after i.m. administration. Propositions for future studies are reviewed. (©Elsevier, Paris)

Résumé — **Traitement médical des crises d'épilepsie induites par les composés organophosphorés.** Les recherches concernant le traitement médical des crises d'épilepsie induites par les composés organophosphorés sont passées en revue. Alors que les antiépileptiques classiquement utilisés en milieu hospitalier s'avèrent inefficaces face au soman, les antagonistes des récepteurs muscariniques sont capables de prévenir ou d'arrêter des crises épileptiques jusqu'à 5 min après leur déclenchement. Les dérivés benzodiazépiniques peuvent aussi être considérés comme un traitement d'urgence efficace durant les 10 premières min d'un état de mal. Comparativement, les antagonistes NMDA sont capables d'arrêter des crises même s'ils sont injectés 40 min après leur déclenchement. Les composés présentant des propriétés mixtes anticholinergiques/antiNMDA pourraient donc s'avérer être les plus prometteurs pour le traitement d'une intoxication organophosphorée. Cependant, leur efficacité après injection intramusculaire, reste à être totalement évaluée. D'autres voies de traitement sont proposées. (©Elsevier, Paris)

soman-induced seizures / NMDA / epilepsy / organophosphate

1. Introduction

Centrally mediated seizures and convulsions are one of the toxic signs that occur following poisoning with organophosphorus (OP) anticholinesterase nerve agents such as soman (pinacolyl methylphosphonofluoridate). Originally, these convulsions were considered a factor that complicated definitive treatment of the more immediate life-threatening effects that nerve agents can have on respiratory function. In the past decade, however, it has become apparent that these seizures rapidly progress to status epilepticus, and contribute to the profound brain damage and cardiac pathology that can develop as a consequence of exposure to these highly toxic compounds. Effective management of nerve agent-induced seizures is critical for both immediate casualty treatment, and minimization of brain and cardiac damage, as well as a rapid and full recovery from the effects of agent exposure.

2. Non-specific management of seizures

In a first approach it seems logical to treat the epileptic syndrome induced by organophosphates

like others epilepsies observed in hospitals. Tonic clonic status epilepticus can be defined as a condition in which prolonged or recurrent tonic clonic seizures persist for 30 min or more. Schematically, two main phenomena could be described in the genesis of neuronal hypersynchron activities with high frequencies: firstly, intrinsic neuronal phenomena leading to an hyperexcitability of neurons and to the generation of repetitive discharges of high frequency action potentials. This could be constituted by perturbations of the intracellular machinery and by changes of the properties or of the structure of neuronal membranes (particularly of complex proteins constituting ionic trans-membrane channels); secondly, inter-neuronal phenomena with a failure of the inhibitory GABAergic transmission and the stimulation of excitatory transmission.

2.1. Classical treatments

Classical treatments in hospital of status epilepticus is founded on the use of several drugs acting on neuronal membranes and modifying in a general manner the synaptic transmission. This leads to an attenuation of the formation and the propagation of abnormal excitations. These compounds have no di-

rect effect on the pathological processes underlying the epilepsy, i.e., in our case, no direct effect on the neurochemical mechanisms involved in the toxicity of soman. Classically used drugs to manage epilepsy are barbiturates, hydantoins, and valproate [1]. Benzodiazepines will not be included in this section since these products may be considered as acting on a specific target involved in the toxicity of organophosphates, i.e., the GABAergic system.

Phenobarbital, pentobarbital, diphenylhydantoin, carbamazepine, valproate have been tested against soman [3, 4]. None of these drugs were effective in preventing soman-induced convulsions. Barbiturates were only effective at anesthetic doses (40 mg/kg and above). It thus appears that drugs classically used against epilepsy in hospital are totally ineffective against organophosphate intoxication in both preventive and curative treatments.

2.2. Use of additional drugs

Additional therapies are classically used during the treatment of generalized seizures in humans. Among these drugs only calcium channel blockers have been tested (flunarizine, nifedipine, verapamil) without success against convulsions induced by soman [2, 5, 6].

3. Specific management of soman-induced seizures

Due to the relative lack of activity of classical antiepileptic drugs (see above), it is important to use specific medications acting on a precise target involved in the development of seizures under soman. There is thus a need to firstly describe the precise

mechanisms, known until now, underlying the toxicity of organophosphates (*figure 1*).

Nerve agent induced seizures are thought to be initiated by large increases in brain acetylcholine that occur after the inhibition of brain acetylcholinesterase by the agents [7, 8]. Increases in brain acetylcholine are capable of triggering recurrent seizure activity in susceptible brain circuits. If left unchecked, seizures rapidly recruit other neurotransmitter systems. The two neurotransmitter systems most critical to this second set of events appear to be the inhibitory GABA system and those involving the excitatory amino acids. Concerning the GABA system, there is a transient impairment of the GABA_A receptor function at the beginning of seizures [9], suggesting that a temporary decrease of GABAergic function may contribute to the early development of seizures (first 10 min). Recent work also emphasized the role of the glutamatergic system in the control of nerve agent-induced seizures. During soman poisoning ACh at a certain threshold triggers release of glutamate, since this release is under control of cholinergic cells [10, 11]. Glu concentration reached a maximal and sustained value in limbic areas after 70 min of seizure [12]. Glu release induces a rapid activation of AMPA receptors during the first 15 min of seizures leading then to a delayed, sustained activation of NMDA receptors in hippocampus [13]. In our opinion AMPA receptors are involved in the early propagation of seizures in combination with muscarinic cholinergic receptors (first 10 min of seizures). Our recent work suggests that AMPA and cholinergic receptors are activated firstly sequentially in the onset and the first 5 min of seizures and then in parallel during the propagation [14]. Then NMDA receptors are involved in the

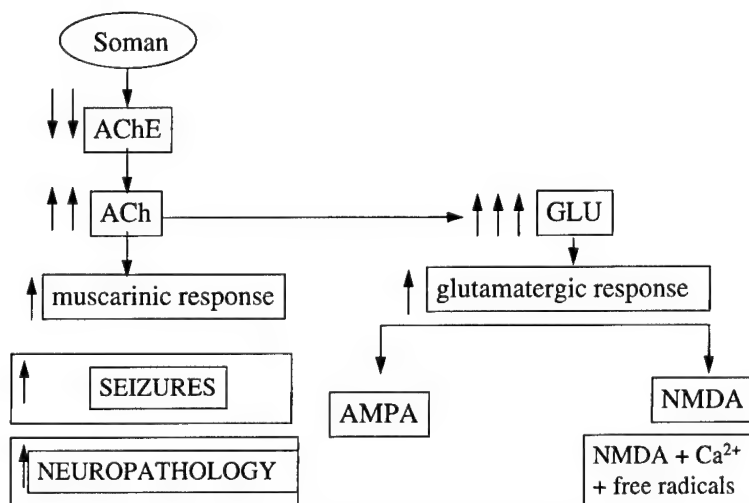


Figure 1. Neurochemical events underlying organophosphorus-induced seizures and neuropathology.

maintenance of epileptic activity and the apparition of subsequent neuropathology [14].

First of all, anticholinergic drugs will be presented. According to the sequence of events presented above, it seems logical to think that anticholinergic drugs will act during the genesis and the early propagation of seizures. Recent work with EEG recordings of McDonough and Shih [15] and Bodjarian et al. [16] demonstrate the ability of atropine to prevent or to stop seizures during the first 5 min of epileptic activity. Conversely, when given at a high dose after 10 min i.m., atropine fails to stop seizures [16, 17]. The anticonvulsant efficacy of atropine as measured by its ED₅₀ against soman was evaluated in [15]. Administered 5 min after seizure onset the ED₅₀ was about 3 mg/kg i.v. while it reaches 87 mg/kg after 20 min of seizures. Similar result were obtained with scopolamine. When scopolamine treatment was delayed for 20 min after seizure onset the ED₅₀ for termination of epilepsy increased 100-fold compared to the value obtained 2–5 min after seizure onset. Based on these results, we assumed that 5 min of seizure activity is representative of the early cholinergic phase of seizure control. Management of seizures after 10 min of paroxystic activity with only anticholinergic drugs thus appears very difficult or impossible. The requirement for progressively higher drug doses, and the eventual failure of anticonvulsant activity by scopolamine and atropine at longer seizure durations could be due to several factors. First, greater drug doses may be required at longer seizure times to compete at the receptor with the progressively greater concentrations of endogenous acetylcholine that occurs after soman intoxication. Alternatively, or in conjunction with this first factor, it is possible that neuronal function has become critically compromised at the longer seizure times due to seizure-induced neuropathology and/or muscarinic receptor loss. A third alternative is that at the longer durations the seizure process is no longer maintained by cholinergic mechanisms.

In summary, muscarinic receptor blockers appears to be able to prevent or stop seizures within only the first 5 min of seizures. There is thus a necessity to develop other anticonvulsants able to counteract soman-induced seizures either during the genesis, the propagation or the maintenance of epileptic activity. Indeed, since the circumstances of poisoning cannot be predicted and may lead to delayed therapy, the search for drugs capable of arresting soman seizures after they are well-established still remains a crucial problem for the teams involved in medical management of OP intoxication.

The second purpose will focus on benzodiazepine products. Benzodiazepines are classically used in the treatment for status epilepticus in the hospital field.

This type of molecule is used in first treatment before barbiturates, hydantoin, carbamazepine. We know that the GABAergic system is involved during the first 10 min of seizures since a decrease of GABAergic function has been observed [8]. It seemed thus logical to investigate the ability of benzodiazepine to counteract soman toxicity by enhancing GABA activity. Information from the reports of Lipp [18, 19], Rumps et al. [20, 21], Rumps and Grudzinska [22] and subsequently supported by the results of Lundy and co-workers [23, 24], Martin et al. [25] and others has indicated the efficacy of diazepam, a commercially available benzodiazepine anticonvulsant, in the treatment of OP poisoning, including soman. In 1991, Shih et al. demonstrated that benzodiazepines like diazepam, midazolam, clonazepam, lorazepam and alprazolam were ineffective in preventing soman-induced convulsions (observation of clinical signs) when used without adjunction of atropine [2]. A more recent study, done with EEG recording, demonstrated that diazepam injected alone i.v. was able to stop seizures induced by soman when used after 5–10 min of seizures [15]. However, for a large proportion of animals where diazepam was initially successful in stopping seizures, there was a reoccurrence of sustained seizure activity after varying periods of initial anticonvulsant effect. Globally the ability of benzodiazepines injected alone to counteract soman induced seizure activity seems limited to the first 5–10 min of status epilepticus thus confirming the transient participation the GABAergic system in the genesis of seizures. McDonough and Shih tested the ability of diazepam to stop seizures 20–40 min after their onset by using the benzodiazepine in conjunction with scopolamine i.v. [15]. Their results demonstrate that in these conditions diazepam continued to exert pronounced anticonvulsant effects at seizure durations of 20 min. However, at 40 min of seizure only four out of six animals stopped seizure and paroxystic activity reoccurred in one of these four animals. The anticonvulsant effectiveness of diazepam thus diminished significantly as a function of seizure duration. In an other study, Shih et al. also demonstrated a potentiation of the effect of diazepam by atropine sulfate. As the doses of atropine sulfate increased, the anticonvulsant ED₅₀ for diazepam decreased [2].

Altogether, it thus appears that: a) benzodiazepine could be considered as an emergency treatment useful before or during the first 10 min of seizures in combination with atropine; and b) that there is an obvious necessity to develop new anticonvulsants able to stop seizures after 30–40 min. Indeed, it appears that the normal inhibitory function of the GABA system is overwhelmed by the massive stimulation and that the neurochemical events maintai-

ning seizure activity are not directly affected by the benzodiazepine products.

In this third part, the recent results obtained with drugs modulating the glutamatergic system will be given. Indeed, if the seizures are allowed to progress unchecked, epileptic process per se, via excessive neuronal depolarization, results in a high release of glutamate that activates AMPA and then NMDA receptors, which can respectively propagate and maintain seizure activity independent of the initial cholinergic activation [26].

In view of the development of a delayed treatment able to stop seizures 40–60 min in duration, EAA antagonists that act at the NMDA receptor, such as MK-801, TCP and procyclidine, have been shown to be effective anticonvulsants when given before or after initiation of nerve agent-induced seizures [2, 15, 27–30]. Studies over the past 6 years have focused on the use of non-competitive NMDA antagonists to counteract soman intoxication because several selective compounds were available which readily crossed the blood-brain barrier (BBB) and were shown to provide good anticonvulsant and neuronal protection (for example dizocilpine or TCP). Competitive antagonists were also chosen because their blocking action could not be overcome by high levels of glutamate released in synapses. The main results obtained with TCP were the following:

1) In the absence of previous atropinization, no clear protection against seizure and neuropathology produced by soman was obtained when a preventive single dose of TCP was administered in rodents. On the other hand, when administration of TCP was repeated once 1 h after soman injection, the anticonvulsant and neuroprotective activity of the anti-NMDA compound became maximal [31]. Taken together, this indicates that: a) neuroprotection by TCP is closely related to the anticonvulsive activity; b) a long, lasting blockade of the NMDA-receptor by the non-competitive inhibitor is necessary for suppressing soman-induced convulsions and related neuropathology; and c) that presence of atropine in the brain is not an absolute prerequisite for TCP to express its beneficial activity on soman poisoning, provided the NMDA-receptor blocker is maintained sufficiently long at suitable cerebral concentration.

2) The presence of a centrally acting muscarinic blocker, potentialized the anticonvulsant properties of TCP [27]. Thus, in conjunction with pretreatment consisting of pyridostigmine and anticholinergic therapy, TCP given 15 min prior to soman did not significantly prevent the onset of seizure activity. However, status epilepticus completely aborted within 30–40 min in all the animals tested. At the same time maximal beneficial effect on survival and clinical recovery were obtained. The inability of TCP

to prevent the appearance of electrographic seizure manifestations before 30–40 min is not surprising if we consider the voltage-dependent mode of binding of PCP-like drugs on NMDA-receptor. TCP acts as an 'open channel blocker'. Its maximal binding is only obtained when PCP sites inside the associated ion channels are sufficiently accessible through depolarization, increase in concentration of glutamate and relief of Mg^{2+} blockade.

3) Due to the 'prerequisite' period of 30–40 min it is therefore not surprising to observe a better activity of TCP when used to terminate ongoing seizures than as a preventive treatment.

4) We have observed that soman-induced seizures became more difficult to arrest with TCP after 90 min of seizures suggesting action of unknown worsening factors.

Similar results were obtained by American groups showing that MK-801, another non-competitive NMDA antagonist, was effective in terminating seizures that had progressed for 20 or 40 min. This was observed in the presence of a pretreatment with scopolamine [15]. It is important to mention that the administration of an antimuscarinic drug is necessary to protect animals from the respiratory depressant effects produced by the interaction between soman intoxication and the non-competitive NMDA antagonist. Indeed, whatever the drug used, TCP or MK-801, an acute respiratory depression or a respiratory arrest, is observable in all intoxicated animals [15, 27]. This respiratory depression disappeared in the presence of atropine or scopolamine. In fact, that TCP and MK-801 may profoundly influence respiration is not surprising since the Glu system plays a pivotal role in central respiratory control and anti-NMDA drugs can elicit apneustic respiration through drastic changes in brainstem rhythmogenesis. However, how atropine sulfate or scopolamine may counteract the deleterious effect of TCP or MK-801 on central respiration. How co-administration of TCP or MK-801 and atropine sulfate may reverse the detrimental activity of soman on central and peripheral respiration remains to be understood.

In summary, NMDA antagonists are capable of terminating soman-induced seizures even if treatment is delayed 40 min after seizure onset. However, they must be used in combination with the anticholinergic drug both to assure a maximal antiepileptic capacity and to prevent a profound respiratory depression.

According to these data, research has also focused on several anticholinergic compounds blocking the toxic effect of NMDA in vitro and in vivo. These drugs thus possess NMDA antagonist activity as well as anticholinergic properties [32]. Such compounds potentially possess the ideal pharmacological proper-

ties for treatment of nerve agent seizures especially at long treatment delays. These agents including trihexyphenidyl, biperiden and benactyzine. Trihexyphenidyl and benactyzine have been tested by Usamricd in 1993 [15]. They were injected i.v. in intoxicated rats after 2–5 min to 40 min of seizure. Both compounds were capable of terminating soman-induced seizures even after 40 min of seizure activity. The termination of soman-induced seizures by benactyzine and trihexyphenidyl at the long seizure duration (20–40 min) in many respects resembled the anticonvulsant effects provided by the combination of scopolamine and MK-801 at the same time. The anticonvulsant activity of trihexyphenidyl and benactyzine against soman-induced seizures at these longer seizure durations may thus be ascribed to mixed anticholinergic and NMDA antagonistic properties of this drug. In 1991 [2], biperiden, trihexyphenidyl and benactyzine injected 30 min before soman were also shown to prevent the onset of soman-induced seizures with ED₅₀ similar to that of scopolamine (0.10 to 0.2 mg/kg). It thus appears that the drugs with both antimuscarinic and anti-NMDA properties may represent the best adequate of a pharmacological treatment to treat soman-intoxication. However, the efficacy of these compounds injected i.m. and the neuroprotective effects of these drugs remain questionable. Indeed, in a recent study, McDonough et al. [3] investigated the ability of biperiden, benactyzine and trihexyphenidyl, injected in conjunction with atropine sulfate i.m., to counteract seizures when injected 5 or 40 min after their onset. It appears, for each drug, that, after 40 min of seizures, the ED₅₀ for termination of epilepsy increased 20- to 40-fold compared to the value obtained 5 min after seizure onset. It is thus clear that the ability of mixed anticholinergic- anti-NMDA compounds, injected i.m., decreases as a duration of the epileptic activity.

4. Conclusion

Drugs classically used against epilepsy in hospital are totally ineffective against organophosphate intoxication.

Muscarinic receptor blockers appear to be able to prevent a stop seizures only within the first 5 min of seizures. There is thus a necessity to develop other anticonvulsants able to counteract soman-induced seizures either during the genesis, the propagation or the maintenance of epileptic activity.

Benzodiazepine could be considered as an emergency treatment useful before or during the first 10 min of seizures in combination with atropine.

NMDA antagonists are capable of terminating soman-induced seizures even if treatment is delayed 40 min after seizures onset. However, they must be used in combination with an anticholinergic drug both to assure a maximal antiepileptic capacity and to prevent a profound respiratory depression.

The development of an 'ideal' antiepileptic drug against soman must take in consideration two specific targets, i.e., muscarinic, NMDA receptors. In this view, drugs with both antimuscarinic and anti-NMDA properties may represent the most adequate pharmacological treatment to treat soman-intoxication. However, the efficacy of these compounds injected by i.m. route and the neuroprotective effects of these drugs remains questionable.

References

- [1] Delgado-Escueta et al., *N. Engl. J. Med.* 306 (1983) 1337–1340.
- [2] Shih et al., *Neurosci. Biobehav. Rev.* 15 (1991) 349–362.
- [3] McDonough et al., *NATO RSG III*, September, 1995.
- [4] Dretchen et al., *Toxic. Appl. Pharmacol.* 83 (1986) 584–589.
- [5] Carpentier et al., *SSA Trav. Sci.* 11 (1990) 85–86.
- [6] Milovanovic et al., *IVGoslav. Physiol. Pharmacol. Acta* 25 (1989) 99–100.
- [7] Lallement et al., *NeuroToxicology* 13 (1992) 557–568.
- [8] Shih, *Psychopharmacol.* 78 (1982) 170–175.
- [9] Lallement et al., *Brain Res.* 629 (1993) 239–244.
- [10] Lallement et al., *Neurosci. Lett.* 139 (1992) 104–107.
- [11] Denoyer et al., *Brain Res.* 592 (1992) 157–162.
- [12] Lallement et al., *Brain Res.* 563 (1991) 234–240.
- [13] Lallement et al., *NeuroToxicology* 12 (1991) 655–664.
- [14] Lallement et al., *Brain Res.* 618 (1993) 227–237.
- [15] McDonough and Shih, *Neurosci. Biobehav. Rev.* 17 (1993) 203–215.
- [16] Bodjarian et al., *NeuroReport* 4 (1993) 1191–1193.
- [17] Capacio and Shih, *Epilepsia* 32 (1991) 604–315.
- [18] Lipp, *Electroencephalogr. Clin. Neurophys.* 32 (1972) 557–560.
- [19] Lipp, *Arch. Int. Pharmacodyn.* 202 (1973) 244–251.
- [20] Rump et al., *Acta Nerv. Super.* 14 (1972) 176–177.
- [21] Rump et al., *Neuropharmacology* 12 (1973) 813–817.
- [22] Rump and Grudzinska, *Arch. Toxicol.* 32 (1974) 223–232.
- [23] Lundy et al., *Arch. Int. Pharmacodyn.* 234 (1978) 64–73.
- [24] Lundy and Shaw, *Neuropharmacology* 22 (1983) 55–63.
- [25] Martin et al., *Brain Res.* 325 (1985) 287–289.
- [26] Lallement et al., *NeuroReport* 5 (1994) 425–428.
- [27] Carpentier et al., *NeuroToxicology* 15 (1994) 837–852.
- [28] Braitman and Sparenborg, *Brain Res. Bull.* 23 (1989) 145–148.
- [29] Shih, *Epilepsy Res.* 7 (1990) 105–116.
- [30] Sparenborg et al., *Neuropharmacology* 31 (1992) 357–368.
- [31] Lallement et al., *Brain Res.* 618 (1993) 227–237.
- [32] McDonough and Shih, *Pharmacol. Biochem. Behav.* 51 (1995) 51, in press.

Treatment of organophosphate poisoning. Experience of nerve agents and acute pesticide poisoning on the effects of oximes

M. Balali-Mood, M. Shariat

Medical Toxicology Centre, Imam Reza Hospital, Mashhad University of Medical Sciences, Mashhad 91735-348, Iran

Abstract — Organophosphate (OP) compounds have been used as pesticides and in chemical warfare (nerve agents). Two nerve agents, tabun and sarine, were used by the Iraqi army against Iranian troops and innocent people. Hundreds of the exposed combatants died in the field. Atropine sulphate has been used successfully in large doses to counteract the muscarinic effects of OP poisoning. The effects of oximes in human OP poisoning have not been well studied. Our aim was to study the effects of obidoxime and pralidoxime in OP pesticide poisoning. The patients were divided into three groups: atropine (A), obidoxime + atropine (OA) and pralidoxime + atropine (PA). Sixty-three patients (33 males, 30 females) with a mean age of 25 years were studied in different groups (43 A, 22 OA and 8 PA). There were no statistical significant differences in major clinical findings and acetylcholinesterase (AChE) activity on admission between the groups. Significant changes were observed during the treatment. Notwithstanding the severity of intoxication – particularly respiratory complications were more observed in the OA and PA groups – there were no fatalities in the PA group, whereas 4 (9%) and 6 (50%) patients in the A and OA groups died, respectively. AChE reactivation was only observed in the PA group, although it was not statistically significant ($r = 0.4747$). There was a good relationship between the AChE reactivation and outcome of the patients. High doses of obidoxime (8 mg/kg followed by 2 mg/kg/h) were found to be hepatotoxic and should be avoided. High doses of pralidoxime (30 mg/kg followed by 8 mg/kg/h) did not induce serious side effects and may be effective in some OP pesticides poisoning. (©Elsevier, Paris)

Résumé — **Traitement des intoxications par organophosphorés. Effet des oximes dans le cas d'intoxication par gaz de combat et pesticides.** Les composés organophosphorés (OP) ont été utilisés comme pesticides et comme gaz de combat (agents neurotoxiques). Deux gaz de combat, appelés tabun et sarin, ont été utilisés par l'armée iraquienne contre les troupes iraniennes et des populations civiles. Des centaines de combattants ont péri sur le champ de bataille. Le sulfate d'atropine à fortes doses a été utilisé avec succès pour supprimer les effets muscariniques des organophosphorés. L'effet des oximes n'a pas été bien étudié dans le cas d'empoisonnements humains pour ces agents. Nous avons donc entrepris d'analyser des effets de l'obidoxime et de la pralidoxime. Les patients ont été divisés en trois groupes, recevant l'atropine seule (A), l'atropine avec l'obidoxime (OA) et la pralidoxime avec l'atropine (PA). Soixante-trois patients (33 hommes et 30 femmes) d'âge moyen 25 ans, ont été traités (43 A, 12 OA et 8 PA). Il n'y avait aucune différence statistiquement significative entre les états cliniques et les niveaux d'acétylcholinestérase (AChE) entre les groupes lors de leur admission, mais des différences importantes sont apparues au cours du traitement. Bien que la sévérité de l'intoxication, en particulier les complications respiratoires, ait été plus marquée dans les groupes OA et PA, il n'y a pas eu de décès dans le groupe PA, alors qu'il y en a eu 4 dans le groupe A (9 %) et 6 dans le groupe OA (50%). Nous n'avons observé de réactivation de l'AChE que dans le groupe PA, et elle n'était pas statistiquement significative ($r = 0,5$). Nous avons observé une bonne corrélation entre la réactivation d'AChE et l'état des patients. De fortes doses d'obidoxime (8 mg/kg, suivi par 2 mg/kg/h) ont produit une hépatotoxicité et doivent être évitées. De fortes doses de pralidoxime (30 mg/kg, suivi de 8 mg/kg/h) n'ont pas induit d'effets secondaires sérieux et peuvent être efficaces dans certains cas d'intoxication par les OPs. (©Elsevier, Paris)

organophosphate / oxime / nerve agent / pesticide / poisoning

1. Introduction

Organophosphate (OP) compounds have largely been used as pesticides in many parts of the world and were also applied in chemical warfare (nerve agents). During the Iraq-Iran war, several chemical attacks were made by the Iraqi army against the Iranian troops and even innocent people. Between August 1983 and March 1984, among the chemical warfare, a nerve agent called tabun was found in the environmental samples and in the patients who were severely intoxicated and died soon after exposure [5]. Toxicological analyses of the blood, urine, skin, hair and gastric juice in 1986 revealed sulfur mustard and tabun [7]. Later in 1987 and 1988, particularly

during the Halabjah massacre, another nerve agent called sarine was also identified.

At the beginning of the chemical warfare attacks, particularly with tabun in early 1983 at Majnoon island, due to the lack of physical and chemical protections, hundreds of the Iranian combatants died in the field. Mortality was much lower later, after using protective clothing, masks and first-aid treatments including auto-injectors containing atropine sulphate.

OP pesticide poisoning has also been a health problem in Iran due to the lack of control on their importation, sale and safe use. Self-poisoning by OP pesticides has been a common cause of admission to the Mashhad Poison Treatment Centre [1] with high morbidity and mortality. Atropine sulphate has

been used successfully in large doses to counteract the muscarinic effects of OP poisoning. The effects of oximes in human OP poisoning have not been well studied. A retrospective study on the effects of oximes in OP pesticide poisoning [2] and a report from Sri Lanka [4] revealed no therapeutic effects. We therefore planned to carry out a prospective clinical trial on the effects of obidoxime and pralidoxime in OP pesticide poisoning.

2. Patients and methods

Patients (aged 14–60 years) with history of oral ingestion of a known OP pesticide who were admitted within 6 h and revealed clinical features of moderate to severe poisoning were investigated. Patients with previous severe organ disorders and those of mixed poisoning (except for the solvent of pesticide) were excluded.

Clinical features on admission and at regular intervals (2, 6, 12, 24 h and then daily) were recorded on a designed form for each patient. Following emergency decontamination and resuscitation on admission (if required), blood samples were taken for the estimation of acetylcholinesterase (AChE) and for the relevant biochemical and haematological investigations at certain intervals as clinically indicated. AChE was estimated in red blood cells (RBC) by a spectrophotometric method using the modified Ellman method [6].

The patients were divided into three groups. The first group received atropine and the other supportive and symptomatic standard treatment. The second and third groups were treated as the first plus oxime infusion as summarised in *table I*. Loading doses of oximes were given over 5 min followed by constant infusion (maintenance) in normal saline until atropine was discontinued. Atropine (IV) administration was given rapidly initially to induce mild to moderate atropinisation (dry mouth, tachycardia, flushing and midriasis within 30 min). It was then continued in constant infusion to maintain atropinisation and when possible in descending manner, based on clinical improvement until discharge. The patients who were severely intoxicated and required mechanical respiration were transferred to ICU for the period of critical care therapy. The

other patients were treated in the ward.

The major clinical and paraclinical parameters including twitching and convulsions, respiratory arrest, required mechanical ventilation and ICU therapy, the rate of AChE reactivation, changes in ECG, total atropine dose used, hospitalisation days and mortality rate of the three groups were compared using standard statistical analyses (Chi-square and Student's *t*-tests) by the Statistical Package for Social Sciences (SPSS). The numerical results are expressed as mean \pm standard deviation.

3. Results

A total of 63 patients (33 male and 30 female) with ages of 25.1 ± 10.4 years were studied. More than 10 OP pesticides that had been taken by the patients were identified. The most common OP pesticides that were involved in different groups and the major clinical findings and AChE activity on admission in each study group are summarised in *tables II* and *III*.

There were no statistical significant differences in the major clinical findings and AChE activity on admission between the groups. However, there were statistically significant differences between the groups on recurrent twitching and convulsions, repeated respiratory arrest, required mechanical ventilation and ICU therapy, hospitalisation days and fatality (*table IV*).

Although the severity of intoxication, based on respiratory complications and hospitalisation days, was higher (< 0.05 to < 0.005) in the pralidoxime group, no mortality was found in this group whereas 4 (12%) and 6 (50%) patients died in the atropine and obidoxime groups, respectively (*table IV*). Three of the patients who received obidoxime developed hepatitis and two of them died due to the liver insufficiency. AChE activities of the atropine and obidoxime groups decreased from 1.7 ± 1.4 and 3.1 ± 2.7 U/mL of packed cells (PC) on admission to 0.8 ± 0.3 and 2.7 ± 1.3 U/mL of PC on the 10th day after admission, whereas it was increased from 1.7 ± 1.6 to 2.6 ± 1.1 U/mL of PC in the pralidoxime group over the same period. Although the AChE reactivation was not statistically significant in the pralidoxime group, there was a significant difference ($P < 0.05$) between the mean slopes of the AChE reactivation curves in the atropine and pralidoxime groups (*figure 1*).

Table I. Treatment regimens of oximes (IV).

Oximes	Loading dose (mg/kg)	Maintenance (mg/kg/h)
Obidoxime (Toxogonon®)	8	2
Pralidoxime (Contrathion®)	30	8

Table II. The most common OP pesticides that had been taken intentionally by the patients.

Groups	Atropine alone	Atropine + obidoxime	Atropine + pralidoxime
Diazinon	+	+	+
Oxydemethon methyl	+	+	–
Malathion	+	+	–
Ethion	+	+	–
Azinphos methyl	+	–	+

Table III. Summary of major clinical findings and acetyl cholinesterase (AChE) activity on admission in different groups.

Findings	Groups	Atropine alone	Atropine + obidoxime	Atropine + pralidoxime
No. of Patients		43	12	8
Sex (M/F)		24/19	6/6	3/5
Age (mean \pm S.D.)		25 \pm 10.6	26.4 \pm 12	23. \pm 7.9
No. of Patient with:				
Twitching		20	5	3
Convulsion		1	2	1
Coma (grade 2–4)		11	6	4
Respiratory arrest		6	1	3
QTc prolongation (> 0.50 s)		12	3	3
AChE ^a (mean \pm S.D., U/mL of PC)		1.7 \pm 1.4	3.1 \pm 2.7	1.7 \pm 1.6

^aNormal, > 4.2 u/mL of PC.

Table IV. Significant changes on the severity of OP intoxication during treatment and outcome of the patients.

Groups	Atropine alone	Atropine + obidoxime	Atropine + pralidoxime	P-value
No. of patients	43	12	8	–
Recurrent twitching and convulsions	4	2	3	0.005
Repeated respiratory arrest	2	3	4	< 0.005
Required mechanical respiration	2	4	2	< 0.05
Required I.C.U. therapy	7	4	5	< 0.05
Hospitalisation days (mean \pm SD)	6.1 \pm 3.7	7.5 \pm 4.3	14 \pm 10	< 0.05
Fatal cases	4	6	0	< 0.005

Atropine doses were given on admission to induce mild to moderate atropinisation (*table V*). Also, 24 h and total doses, together with the total administered oxime doses, are summarised in *table V*.

4. Discussion

Although the severity of OP intoxication on admission in the three groups was similar based on clinical findings and AChE activities (*table III*), significant changes were observed during the treatment (*table IV*). Due to the limited number of the cases studied so far in the pralidoxime group, it is difficult to make a good judgment. However, it is of interest that there was no fatality in the pralidoxime groups, whereas the mortality rate was very high (50%) in the patients receiving obidoxime. There is no doubt that the high dose of obidoxime used increased mortality, particularly due to the hepatotoxicity which was only observed in this group. Liver

dysfunction and hepatotoxicity of obidoxime has already been considered by Bismuth et al. [3].

Unfortunately there has been no control on the importation, distribution and safe use of OP pesticides in Iran. Thus, a very wide range of them are almost freely used in the country. More than 10 different OPs were taken by the patients as self-poisoning in this study. Therefore, the number of patients who had taken one particular OP in each group was not enough for statistical comparison. However, the study is continuing and we may reach a conclusion in the future.

It seems that the reactivation of AChE is an important factor for the patient's recovery as judged by the 100% recovery in the pralidoxime group, in which the AChE reactivation occurred (*figure 1*). The anticonvulsant activity and the anticholinergic effects of oximes that were reported [9, 10] were not observed in this study. In fact the atropine doses required for atropinisation were higher rather than lower in the oxime groups (*table V*).

Table V. Administered atropine and oxime doses (mean \pm S.D.).

Groups	Atropine alone	Atropine + obidoxime	Atropine + pralidoxime
Initial atropine dose (mg)	51 \pm 39	74 \pm 75	180 \pm 335
24 h of doses of atropine (mg)	123 \pm 109	343 \pm 362	295 \pm 425
Total atropine doses (mg)	504 \pm 1553	701 \pm 571	542 \pm 525
Oxime doses (g)	–	14.0 \pm 7.4	60.6 \pm 24.3

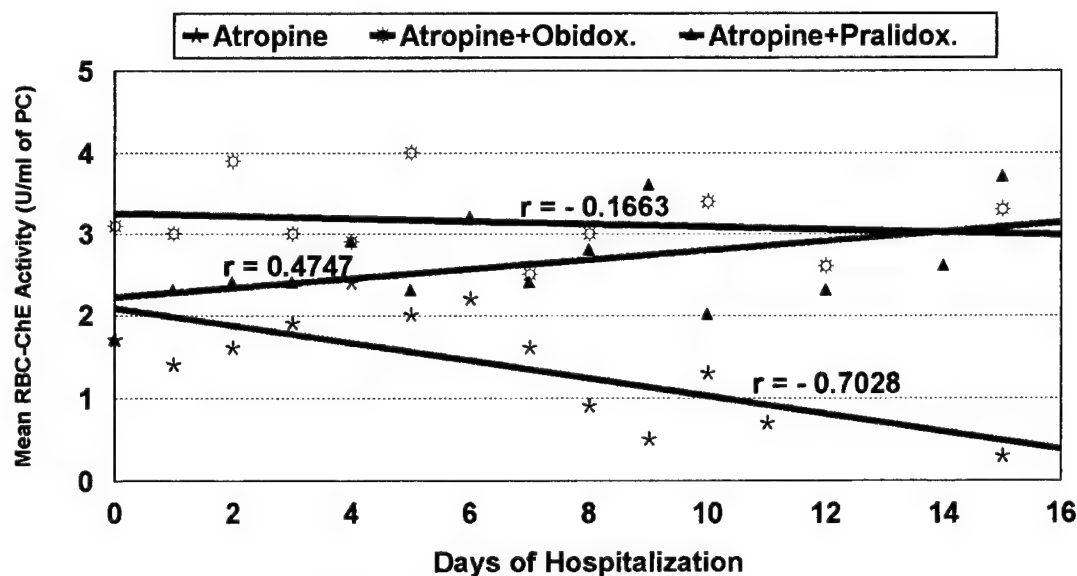


Figure 1. Mean acetyl cholinesterase (RBC) activity in the acute organophosphate poisoning.

High doses of pralidoxime revealed no serious side effects and significantly improved the patients outcome. It has also been used in children successfully without significant side effects in the Mashhad Poisons Treatment Centre and elsewhere [8]. Since it has been known that pralidoxime does not cross the blood-brain barrier (whereas obidoxime may do so) and thus can not reactivate the brain AChE, it can be concluded that the inhibition of central AChE is less important as suggested previously [3].

Based on the preliminary results of this study the following conclusions can be drawn: i) high doses of obidoxime may induce hepatotoxicity and can increase morbidity and mortality of OP poisoning. It is thus recommended to avoid using high doses of obidoxime; ii) high doses of pralidoxime revealed no serious side effects and since it reduces the morbidity and mortality, it could be used in OP poisoning; and iii) reactivation of AChE may play an important role for the outcome of OP poisoning.

However, further studies are required to investigate the effects of high doses of pralidoxime and low doses of obidoxime in common known OP poisoning. This is currently undertaken in the Mashhad Poison Treatment Centre of Iran.

Acknowledgment

Financial support of the Iranian Academy of Medical Sciences to this research project is greatly acknowledged.

References

- [1] Balali-Mood M., Shariat M., Pattern of acute poisoning in Mashhad, M. J. Nabz 5 (1995) 13-19.
- [2] Balali-Mood M., Shariat M., Effects of oximes in acute organophosphate pesticide poisoning - a retrospective study, Proceeding of the Chemical and Biological Medical Treatment Symposium, Spiez, Switzerland, 1996, pp. 119-124.
- [3] Bismuth C., Inns R.H., Marrs T.C., Efficacy, toxicity and clinical use of oxime in anticholinesterase poisoning, in: Balantyne B., Marrs T.C., Aldrige W.N. (Eds.), Clinical and experimental toxicology of organophosphates and carbamates, Butterworth-Heinemann Ltd, Oxford, 1992, pp. 555-577.
- [4] De Silva H.J., Wijewickrama R., Senanayake N., Does pralidoxime affect outcome of management in acute organophosphate poisoning, Lancet 339 (1992) 1136-1138.
- [5] Foroutan A., Report of the specialist appointed by the Secretary-General of the United Nation to investigate allegation by the Islamic Republic of Iran concerning the use of Chemical weapons, Proceeding of the First World Congress on Biological and Chemical Warfare, Ghent, May 21-23, 1984, pp. 302-310.
- [6] George P.M., Abernethy M.H., Improved Ellman procedure for erythrocyte cholinesterase, Clin. Chem. 29 (1983) 365-368.
- [7] Hendrickx B., Report and conclusion of the biological samples of men, intoxicated by war gases, send to the department of toxicology of the State University of Ghent, for toxicological investigation, Proceeding of the Second World Congress on Biological and Chemical Warfare, Ghent, August 24-27, 1986, pp. 553-582.
- [8] Henry C., Farrar M.D., Thoma G, et al., Use of continuous infusion of pralidoxime for treatment of organophosphate poisoning in children, J. Pediatr. 116 (1990) 4.
- [9] Minton N.A., Murray V.S.G., A review of organophosphate poisoning, Med. Toxicol. 3 (1988) 350-375.
- [10] Lotti M., Treatment of acute organophosphate poisoning, Med. J. Aust. 154 (1991) 7.

Influence of retinoic acid and of cyclic AMP on the expression of choline acetyltransferase and of vesicular acetylcholine transporter in NG108-15 cells

Marie-Françoise Diebler, Monique Tomasi, Françoise-Marie Meunier,
Maurice Israël*, Vladimir Doležal**

Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, 91190 Gif-sur-Yvette, France

Abstract — Treatment of the cholinergic cell line NG108-15 with retinoic acid or cAMP results in an increase of choline acetyltransferase activity (ChAT) whereas none of these agents influences the amount of the vesicular acetylcholine transporter (VACHT) as judged from vesamicol binding and immunoblot studies. We suggest that immaturity of posttranslational events controlling the expression of VACHT protein is responsible for the apparent absence of coregulation of ChAT and VACHT protein expression. (©Elsevier, Paris)

Résumé — Influence de l'acide rétinoïque et de l'AMP cyclique sur l'expression de la choline acétyltransférase et du transporteur vésiculaire d'acétylcholine dans les cellules NG108-15. Le traitement des cellules NG108-15 par l'acide rétinoïque ou l'AMP cyclique induit une augmentation de l'activité choline acétyltransférase (ChAT). Toutefois, la quantité de transporteur vésiculaire d'acétylcholine (VACHT), estimée par des mesures de liaison du vésamicol et par détection de la protéine sur immunoblot, n'est pas modulée par ces agents. Nous proposons l'hypothèse que l'absence apparente de corégulation des deux protéines ChAT et VACHT est due à l'immaturité de processus post translationnels régulant l'expression de VACHT. (©Elsevier, Paris)

choline acetyltransferase / vesicular acetylcholine transporter / NG108-15 / retinoic acid / dbcAMP

1. Introduction

A typical feature of cholinergic neurons is the presence of a set of specific proteins which is not found in other neuronal or non-neuronal cells. Two of these proteins, choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VACHT), i.e., the proteins which ensure the synthesis of acetylcholine (ACh) and the translocation and storage of ACh in synaptic vesicles, are essential traits of the cholinergic phenotype. A unique genomic organisation where the intronless VACHT gene is embedded within the first intron of the ChAT gene has recently been discovered in mammals [3, 12] and described as a 'cholinergic locus' [12]. This arrangement, which is conserved from the nematode [1] to man, offers an unusual template for a common regulation at the transcriptional level. Indeed, it has been reported that in primary cultures of sympathetic neurons, the activation of this locus by different treatments led to a phenotypic switch evidenced by a parallel increase in ChAT and VACHT mRNAs [5, 23] and functional ChAT and VACHT proteins [5]. Parallel upregulation of ChAT and VACHT mRNA expression by different

factors has been described in cholinergic SN56 cells and parallel downregulation in PC12 cell line [6, 7, 25].

The cell line NG108-15 (mouse neuroblastoma N18TG-2 × rat glioma C6BU-1) displays traits regarded as characteristic for a cholinergic phenotype. Although the parental cells have no detectable ChAT activity, the hybrid cells were shown to be able to synthesise and release ACh [15] (for recent review, see Zhong et al. [32]) and thus may provide a useful model for studying basic aspects of the function of cholinergic neurons. However, these cells release only little of their endogenous ACh upon stimulation with a calcium ionophore bypassing voltage-operated calcium channels; the amount of ACh released becomes substantially higher after the cells had been loaded with exogenous ACh [18]. After differentiation of the cells with dibutyryl cyclicAMP (dbcAMP), the depolarization activated liberation of endogenous ACh increased [20, 21], whereas the release of loaded ACh activated by calcium ionophore remained unchanged [18]. Taken together it suggests that these cells have fully developed a releasing machinery but that the storage of ACh is defective. In line with this suggestion is the observation that the transfection of NG108-15 cells with cDNA for ChAT enhances ChAT activity and ACh content in the cells but does not increase either spontaneous or electrically evoked ACh release [31].

* Correspondence and reprints

** Permanent address: Institute of Physiology, CAS, 14220 Prague, Czech Republic

In the present work we addressed the question whether differentiation factors known to increase the level of mRNAs for ChAT and VACHT in primary cultures and in cholinergic SN56 cell lines do induce the parallel appearance of functional ChAT and VACHT proteins in NG108-15 cells.

2. Results and discussion

2.1. Effect of retinoic acid and dbcAMP on ChAT activity

Several groups of agents have been shown to modulate ChAT expression on primary neuronal cell cultures and cell lines (for review see [30]). Among them, retinoids and cAMP were shown to enhance ChAT transcription [4, 7, 8, 16, 19, 22]. In the first set of experiments shown in *figure 1*, we tested the effect of retinoic acid and dbcAMP on the activity of ChAT and the synthesis of ACh from labelled choline. Consistent with previous reports on other cell lines, retinoic acid (1 μ M) increased the activity of ChAT in NG108-15 cells (*figure 1A*). This increase was time dependent; it was already apparent after 48 h (2.5-fold increase) and it reached a plateau after 5 to 7 days (5-fold increase). Similarly, a dbcAMP treatment (1 mM) for 5 days also increased ChAT activity, although to a lesser extent (about 3-fold increase). Stimulation of ChAT activity by dbcAMP was concentration dependent (*figure 1C*). The concentration dependence curves showed that ChAT activity is increased at lower concentrations of dbcAMP than acetylcholinesterase (AChE) activity, which was only increased by 50% by 1 μ M retinoic acid (*figure 1B*). Both treatments resulted in an augmentation of the accumulation of labelled ACh in the cells during a 30-min incubation in the presence of labelled choline (*figure 1D*). The extent of increase of labelled ACh content approximated that of ChAT activity. The accumulation of ACh was largely enhanced in controls and in treated cells as well, when an irreversible, permeable inhibitor of cholinesterases (paraoxon) was included in the incubation medium (*figure 1D*).

Our observation indicates that retinoic acid increases the expression of ChAT activity in NG108-15 to a larger extent than dbcAMP. Although we did not analyse the effects of these drugs on mRNAs levels, one can expect that the increased enzymatic activity is a consequence of enhanced translation, as it has been observed in the same cell line with cAMP [16, 22] and in other cell lines with retinoids [7].

2.2. Effect of retinoic acid and dbcAMP on VACHT

In the next experiments we followed the expression of VACHT protein in NG108-15 grown in basal medium or in the presence of retinoic acid or of dbcAMP. We first measured the level of VACHT using a binding assay with vesamicol as a specific ligand [2, 27]. Control cell membranes did bind vesamicol and the binding parameters did not appear to depend on the time in culture between 3–6 days. Pooled values of B_{\max} and K_d in controls were 3.92 ± 0.36 pmol/mg protein and 128 ± 21 nM ($n = 7$), respectively. The dissociation constant of vesamicol binding is rather high compared to values found in synaptic vesicles isolated from *Torpedo* electric organ [9, 24] and in membranes of transfected cells expressing *Torpedo* or rat VACHT [12, 27]. Treatment of the cells with either retinoic acid for 3–6 days or with dbcAMP for 6 days had no significant effect on the binding of vesamicol (*figure 2A,B*). The values for B_{\max} and K_d (*figure 2C, D*) were $91 \pm 20\%$ and $73 \pm 12\%$ ($n = 5$) of paired controls for retinoic acid treatment and $98 \pm 42\%$ and $90 \pm 18\%$ ($n = 2$) for dbcAMP treatment, respectively.

We next evaluated the level of VACHT expression using Western blot analysis. VACHT protein was detected with an immunopurified polyclonal antibody directed against the C-terminal part of the human VACHT [28]. The protein pattern recognised by the antibody was unexpected. Rat and human VACHT have a predicted molecular mass around 56 kDa and are highly glycosylated. They appear on immunoblot as a diffuse and heterogeneous band between 55 and 75 kDa [5, 13, 17, 28]. In NG108-15, the major band which is recognised by the antibody is a peptide with a smaller molecular mass of around 33 kDa, while only faint staining is found around 56 kDa. This small peptide is most probably a proteolytic fragment of the protein as it does not correspond to the N-deglycosylated form of VACHT which appears as a doublet around 39 kDa [29]. A similar peptide pattern was observed using an antibody directed against the C terminus part of the rat VACHT (data not shown). In line with vesamicol binding experiments, neither retinoic acid (*figure 3*) nor dbcAMP (data not shown) increased the level of the peptides which are detected by the antibody. *Figure 3* also shows that the level of synaptophysin, a synaptic vesicle marker, remained unchanged after retinoic acid treatment.

Immunofluorescence studies showed that VACHT staining appears as large dots completely different from the finely punctuate pattern of synaptophysin staining (*figure 4*) and from the pattern of VACHT staining which is observed in transfected N18 and AtT20 cells expressing *Torpedo* VACHT [29].

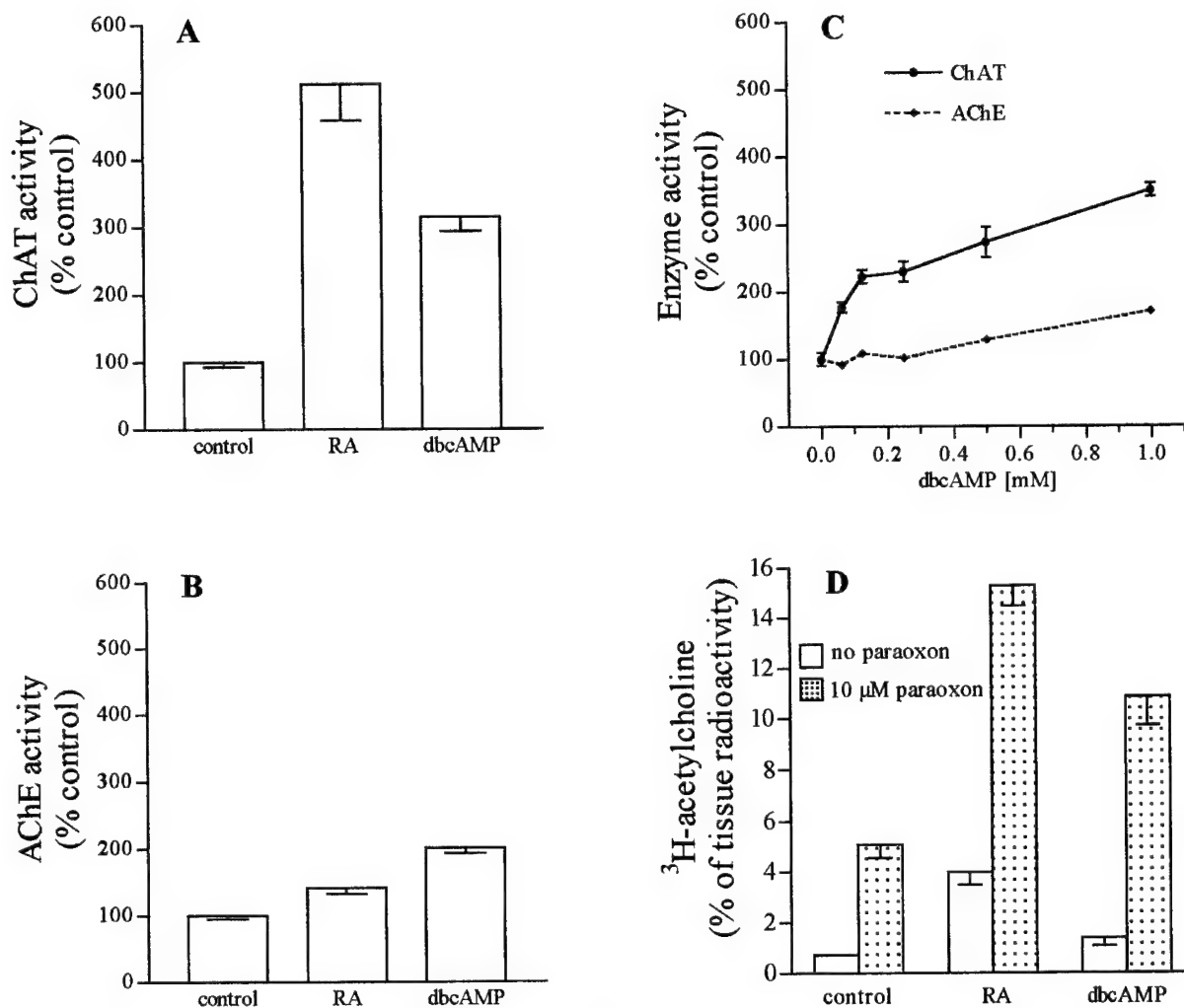
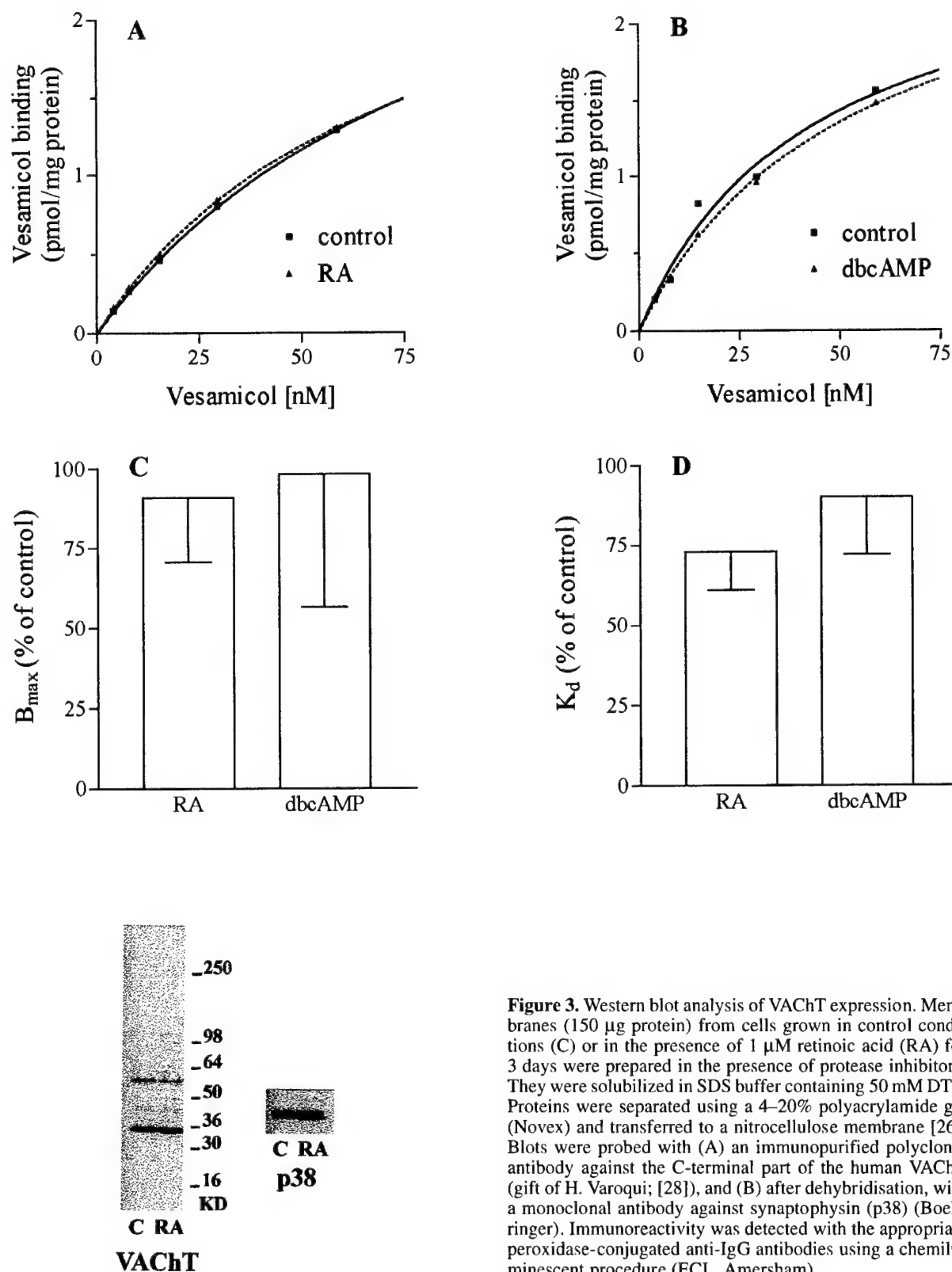


Figure 1. Effect of retinoic acid and dbcAMP on ChAT and AChE activities, and on ACh content. NG108-15 cells were grown for 5 days without addition (control) or in the presence of 1 μ M retinoic acid (RA) or 1 mM dbcAMP. **A, B.** The cells were washed, disrupted in 10 mM sodium phosphate buffer, pH 7.4, and aliquots of this homogenate were used for the determination of ChAT activity (**A**) according to Berrard et al. [5] and AChE activity (**B**) as described by Ellman et al. [11]. **C.** The cells were grown for 5 days in the presence of increasing concentrations of dbcAMP and the activities of ChAT and AChE were assayed. The results in **A–C** are expressed in percentage of corresponding controls ($n = 4–20$). Control value of ChAT activity was 1.09 ± 0.09 nmol of ACh/mg protein \times 15 min ($n = 24$) and that for AChE was 1.92 ± 0.08 of arbitrary units/mg protein \times 20 min ($n = 36$). **D.** The cells were washed and then incubated for 30 min at 37°C in HEPES buffered saline containing tritiated choline either with or without 10 μ M paraoxon. At the end of the incubation they were washed in fresh saline and extracted into 5% TCA. The content of labelled acetylcholine in TCA extract was determined as described by Doležal and Tuček [10] and is expressed in percentage of the total incorporated radioactivity ($n = 2–8$).

Figure 2. Effect of retinoic acid and dbcAMP on the binding of vesamicol. The cells were grown either in control conditions or in the presence of 1 μ M retinoic acid (RA) for 3–6 days or in the presence of 1 mM dbcAMP for 6 days. The binding of vesamicol was measured on membrane preparation as described by Varoqui et al. [27]. **A.** Average curves of vesamicol binding from six independent experiments in controls and after treatment with RA. Kinetics parameters for control are $B_{\max} = 3.48 \pm 0.10$ pmol/mg protein and $K_d = 99.3 \pm 4.1$ nM (mean \pm S.E.M.). **B.** Average curves of vesamicol binding from two independent experiments in controls and after treatment with dbcAMP. Kinetics parameters for control are $B_{\max} = 3.51 \pm 0.46$ pmol/mg protein and $K_d = 71.5 \pm 0.5$ nM (mean \pm S.E.M.). **C, D.** B_{\max} and K_d values expressed in percentage of paired controls.



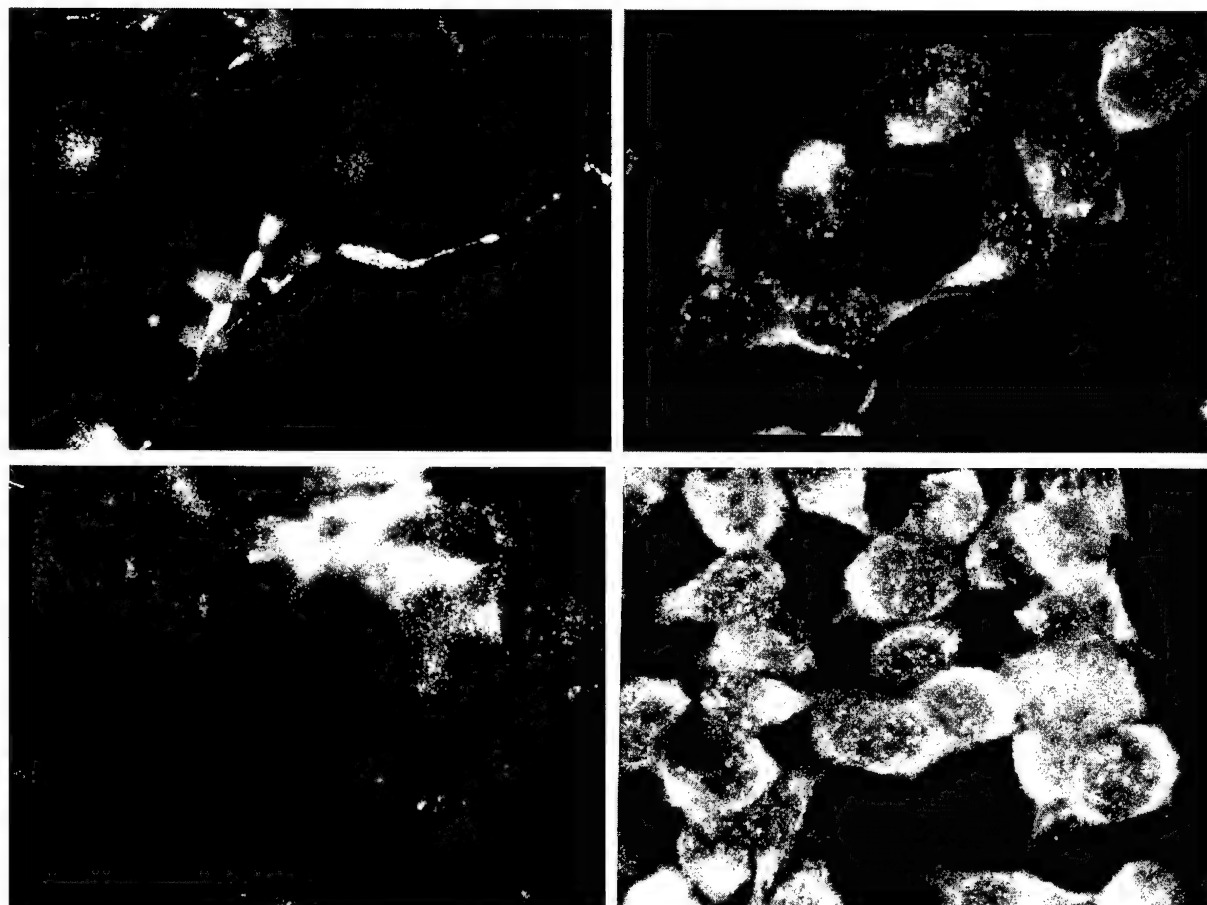


Figure 4. Immunocytochemistry of VAcHt. Cells grown on coated glass coverslips in basal medium (upper part) or in the presence of 1 μ M retinoic acid (lower part) were fixed and permeabilized with cold (-20°C) acetone. They were then incubated with a polyclonal rat VAcHt antibody (right) or with an antisynaptophysin antibody (Boehringer) (left). Binding of primary antibodies was detected with suitable secondary antibodies. Immunofluorescence images were viewed with appropriate filters using an epifluorescence microscope.

3. Conclusions

The effects of cholinergic differentiation agents on cell lines have previously been estimated on the basis of ChAT and VAcHt mRNA levels and ChAT activity. To our knowledge, their effect on the expression of the VAcHt protein has not been reported. The present data indicate that in NG108-15 cells, the VAcHt protein as recognised by antibodies and as judged from vesamicol binding, has unusual characteristics. Preliminary experiments revealed similar results on VAcHt protein in SN56 (gift of B.Wainer; [14]) where transcription of VAcHt is coregulated with that of ChAT [7]. These observations suggest that most of the protein is not correctly processed, either because the cells are unable to perform due posttranslational modifications such as N-glycosylations, and the protein is thus rapidly degraded, or

that the targeting of the protein is compromised. Moreover, it has been reported that during the development of rat brain, there is a parallel increase of ChAT mRNA and ChAT activity whereas the appearance of a mature VAcHt protein as detected on immunoblots lags behind VAcHt mRNA transcription by several weeks [17]. Consistent with these observations, we suggest that hybrid cells as NG108-15 are incapable to undergo sufficient differentiation allowing a proper processing of VAcHt and/or its incorporation into vesicle membrane. The mechanisms which participate in the posttranslational control of the expression of VAcHt are still to be understood.

Acknowledgments

This work was supported by CNRS, AFM, DRET (grant no 95/133) and a fellowship to V. Dolezal from AFM.

References

- [1] Alfonso A., Grundahl K., Duerr J.S., Hahn H.-P., Rand J.P., The *Caenorhabditis elegans unc17* gene: a putative vesicular acetylcholine transporter, *Nature* 261 (1993) 617–619.
- [2] Bahr B.A., Parsons S.M., Demonstration of a receptor in *Torpedo* synaptic vesicles for the acetylcholine storage blocker L-trans-2- (4-phenyl[3,4-³H] piperidino) cyclohexanol, *Proc. Natl. Acad. Sci. USA* 83 (1986) 2267–2270.
- [3] Béjanin S., Cervini R., Mallet J., Berrard S., A unique gene organization for two cholinergic markers, choline acetyltransferase and a putative vesicular transporter of acetylcholine, *J. Biol. Chem.* 269 (1994) 21944–21947.
- [4] Berrard S., Faucon Biguet N., Houhou L., Lamouroux A., Mallet J., Retinoic acid induces cholinergic differentiation of cultured newborn rat sympathetic neurons, *J. Neurosci. Res.* 35 (1993) 382–389.
- [5] Berrard S., Varoqui H., Cervini R., Israël M., Mallet J., Diebler M.-F., Coregulation of two embedded gene products: choline acetyltransferase and the vesicular acetylcholine transporter, *J. Neurochem.* 65 (1995) 939–942.
- [6] Berse B., Blusztajn J.K., Coordinated upregulation of choline acetyltransferase and vesicular acetylcholine transporter gene expression by the retinoic acid receptor alpha, cAMP, and leukemia inhibitory factor/ciliary neurotrophic factor signaling pathways in a murine septal cell line, *J. Biol. Chem.* 270 (1995) 22101–22104.
- [7] Berse B., Blusztajn J.K., Modulation of cholinergic locus expression by glucocorticoids and retinoic acid is cell-type specific, *FEBS Lett.* 110 (1997) 175–179.
- [8] Cervini R., Berrard S., Béjanin S., Mallet J., Regulation by CDF/LIF and retinoic acid of multiple ChAT mRNAs produced from distinct promoters, *Neuroreport* 5 (1994) 1346–1348.
- [9] Diebler M.-F., Morot Gaudry-Talarmin Y., AH5183 and cettidil: two potent inhibitors of acetylcholine uptake into synaptic vesicles from *Torpedo marmorata*, *J. Neurochem.* 52 (1989) 813–821.
- [10] Doležal V., Tuček S., Positive and negative effects of tacrine (tetrahydroaminoacridine) and methoxytacrine on the metabolism of acetylcholine in rat brain cortical prisms incubated under 'resting' conditions, *J. Neurochem.* 56 (1991) 1207–1215.
- [11] Ellman G., Courtney K.D., Andrews J., Featherstone R.M., A new and rapid colorimetric determination of acetylcholinesterase activity, *Biochem. Pharmacol.* 7 (1961) 88–95.
- [12] Erickson J.D., Varoqui H., Schäfer M.K.H., Modi W., Diebler M.-F., Weihe E., Rand J., Eiden L.E., Bonner T.I., Usdin T.B., Functional identification of a vesicular acetylcholine transporter and its expression from a 'cholinergic' gene locus, *J. Biol. Chem.* 269 (1994) 21929–21932.
- [13] Gilmor M.L., Nash N.R., Roghani A., Edwards R.H., Yi H., Hersch S.M., Levey A.I., Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles, *J. Neurosci.* 16 (1996) 2179–2190.
- [14] Hammond D.N., Lee H.J., Tongsgard J.H., Wainer B.H., Development and characterization of clonal cell lines derived from septal cholinergic neurons, *Brain Res.* 512 (1990) 190–200.
- [15] Hamprecht B., Structural, electrophysiological, biochemical and pharmacological properties of neuroblastoma glioma cell hybrids in cell culture, *Int. Rev. Cytol.* 49 (1977) 99–170.
- [16] Hersh L.B., Induction of choline acetyltransferase in the neuroblastoma x glioma cell line NG108-15, *Neurochem. Res.* 17 (1992) 1063–1067.
- [17] Holler T., Berse B., Cermak J.M., Diebler M.-F., Blusztajn J.K., Differences in the developmental expression of the vesicular acetylcholine transporter and choline acetyltransferase in the rat brain, *Neurosci. Lett.* 212 (1996) 107–110.
- [18] Israël M., Lesbats B., Synguelakis M., Joliot A., Acetylcholine accumulation and release by hybrid NG108-15, glioma and neuroblastoma cells – role of 16 kDa membrane protein in release, *Neurochem. Int.* 25 (1994) 103–109.
- [19] Kobayashi M., Matsuoaka I., Kurihara K., Cholinergic differentiation of cultured sympathetic neurons induced by retinoic acid. Induction of choline acetyltransferase-mRNA and suppression of tyrosine hydroxylase-mRNA levels, *FEBS Lett.* 337 (1994) 256–264.
- [20] Kumagai-Tohda C., Tohda M., Nomura Y., Increase in neurite formation and acetylcholine release by transfection of growth-associated protein-43 cDNA into NG108-15 cells, *J. Neurochem.* 61, 526–532.
- [21] McGee R., Simpson P., Christian C., Mata M., Nelson P., Nirenberg M., Regulation of acetylcholine release from neuroblastoma x glioma hybrid cells, *Proc. Natl. Acad. Sci. USA* 75 (1978) 1314–1318.
- [22] Misawa H., Takahashi R., Deguchi T., Transcriptional regulation of choline acetyltransferase gene by cyclic AMP, *J. Neurochem.* 60 (1993) 1383–1387.
- [23] Misawa H., Takahashi R., Deguchi T., Coordinate expression of vesicular acetylcholine transporter and choline acetyltransferase in sympathetic superior cervical neurones, *Neuroreport* 6 (1995) 965–968.
- [24] Norenberg K., Parsons S.M., Regulation of the vesamicol receptor in cholinergic synaptic vesicles by acetylcholine and an endogenous factor, *J. Neurochem.* 52 (1989) 913–920.
- [25] Pedersen W.A., Berse B., Schuler U., Wainer B.H., Blusztajn J.K., All-trans- and 9-cis-retinoic acid enhance the cholinergic properties of a murine septal cell line: evidence that the effects are mediated by activation of retinoic acid receptor-alpha, *J. Neurochem.* 65 (1995) 50–58.
- [26] Towbin H., Staehelin T., Gordon J., Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, *Proc. Natl. Acad. Sci. USA* 76 (1979) 4350–4354.
- [27] Varoqui H., Diebler M.-F., Meunier F.-M., Rand J.B., Usdin T.B., Bonner T.I., Eiden L.E., Erickson J.D., Cloning and expression of the vesamicol binding protein from the marine ray *Torpedo*, *FEBS Lett.* 342 (1994) 97–102.
- [28] Varoqui H., Erickson J.D., Active transport of acetylcholine by the human vesicular acetylcholine transporter, *J. Biol. Chem.* 271 (1996) 27229–27232.
- [29] Varoqui H., Meunier F.M., Meunier F.A., Molgo J., Berrard S., Cervini R., Mallet J., Israël M., Diebler M.F., Expression of the vesicular acetylcholine transporter in mammalian cells, *Progr. Brain Res.* 109 (1996) 83–95.
- [30] Wu D., Hersh L.B., Choline acetyltransferase: celebrating its fiftieth year, *J. Neurochem.* 62 (1994) 1653–1663.
- [31] Zhong Y.G., Kimura Y., Noda M., Misawa H., Higashida H., Discrete acetylcholine release from neuroblastoma or hybrid cells overexpressing choline acetyltransferase into the neuromuscular synaptic cleft, *Neurosci. Res.* 22 (1995) 81–88.
- [32] Zhong Y.G., Misawa H., Furuya S., Kimura Y., Noda M., Yokoyama S., Higashida H., Overexpression of choline acetyltransferase reconstitutes discrete acetylcholine release in some but not all synapse formation-defective neuroblastoma cells, *J. Physiol. (Paris)* 89 (1995) 137–145.

From the cholinergic gene locus to the cholinergic neuron

Eberhard Weihe^a, Martin K.-H. Schäfer^a, Burkhard Schütz^a, Martin Anlauf^a,
Candan Depboylu^a, Christian Brett^a, Liangbiao Chen^b, Lee E. Eiden^b

^a*Department of Anatomy and Cell Biology, Philipps University, Robert-Koch Strasse 6, Marburg 35033, Germany*

^b*Section on Molecular Neuroscience, Laboratory of Cellular and Molecular Regulation, National Institute of Mental Health, NIH, 36 Convent Drive, MSC 4090, Bethesda, MD 20892-4090, USA*

Abstract — The cholinergic gene locus (CGL) was first identified in 1994 as the site (human chromosome 10q11.2) at which choline acetyltransferase and a functional vesicular acetylcholine transporter are co-localized. Here, we present recent neuroanatomical, developmental, and evolutionary insights into the chemical coding of cholinergic neurotransmission that have been gleaned from the study of the CGL, and its protein products VACHT and ChAT, which comprise a synthesis-sequestration pathway that functionally defines the cholinergic phenotype. (©Elsevier, Paris)

Résumé — Du locus génétique cholinergique au neurone cholinergique. Le locus génétique cholinergique (cholinergic gene locus, CGL) a été identifié en 1994 et regroupe les gènes de la choline acétyltransférase et d'un récepteur vésiculaire d'acétylcholine (chromosome humain 10q11.2). Nous présentons ici des données sur la neuroanatomie, le développement et l'évolution de la neurotransmission cholinergique obtenus à partir de l'étude du CGL et de ses produits protéiques, VACHT et ChAT : ce système de synthèse-séquestration définit fonctionnellement le phénotype cholinergique. (©Elsevier, Paris)

synaptic vesicle / acetylcholine / VACHT / ChAT / peripheral and central nervous system / ontogeny / cholinergic differentiation

The cholinergic gene locus (CGL) comprises an approximately 80 kb region of the mammalian genome that encodes choline acetyltransferase (ChAT), the vesicular acetylcholine transporter (VACHT) and the regulatory sequences responsible for expression of VACHT and ChAT in cholinergic neurons. The CGL is so named because it encodes the two proteins that functionally define the cholinergic neuronal phenotype, within a single regulatory domain [16]. Our current view based on extensive analysis of the rat and primate nervous systems is that VACHT and ChAT mRNA and protein are invariably co-expressed, and thus are both adequate markers for the cholinergic phenotype (see [3] for review). VACHT immunohistochemistry offers certain advantages in surveying fixed tissue, and has allowed the unambiguous identification of peripheral muscular vasculature and the hypothalamus in the central nervous system, as targets of cholinergic innervation ([10, 11] and references therein). Most notably, VACHT immunohistochemistry has revealed that noradrenergic and cholinergic phenotypes are co-expressed in developing autonomic neurons of both the parasympathetic and sympathetic lineages. The former become exclusively cholinergic and the latter mainly noradrenergic through a process of phenotypic restriction, rather than noradrenergic/cholinergic 'switching', during development [12, 13]. Improved VACHT immunohistochemistry is perfectly suited to delineate peripheral cholinergic neurons and termi-

nals and to characterize peptidergic cotransmitters. Our recent immunohistochemical and in situ hybridization studies indicate that virtually all enteric neurons of the upper gastrointestinal tract co-express VIP and acetylcholine, suggesting that the 'non-adrenergic/non-cholinergic (NANC)' component of the enteric nervous system is much less extensive than previously believed (Anlauf et al., unpublished observations). VACHT immunoreactivity is present in interneurons of both rodent and primate cerebral cortex ([10] and references therein). In addition, the patterns of cholinergic innervation of the primate cortex, now examined in detail in paraffin-embedded tissues, suggest a decidedly lamina- and area-specific cholinergic innervation. A detailed picture of the cholinergic innervation of the telencephalon provides the foundation for a clearer understanding of cholinergic function in cognition, arousal, and neurodegenerative diseases such as Alzheimer's disease. With respect to the suggested neuroinflammatory background of these diseases it is of interest that the prostaglandin synthesizing enzyme cyclooxygenase 2 (COX-2) is preferentially co-expressed with VACHT in primate cholinergic projection neurons of the basal forebrain (Depboylu et al., unpublished observations).

While expression of both VACHT and ChAT from the CGL is required for the cholinergic phenotype, non-concordance in VACHT and ChAT expression in vivo could function as a mechanism to fine-tune cho-

linergic function. For example, the ratio of VACHT to ChAT mRNA in at least some parts of the peripheral nervous system of the adult rat is higher than in the central nervous system (*figure 1*). This may be related to an early pattern of VACHT overexpression relative to ChAT both centrally and peripherally during development [6] (Schütz et al., unpublished observations). How might the CGL be regulated at the transcriptional level to allow differential expression of these two gene products, and to what purpose? In the nematode, VACHT and ChAT are transcribed from separate messenger RNAs that arise from a common primary transcript, with presumably a single transcriptional start site, via differential splicing [2]. This is also the case in *Drosophila* [7]. However, the ratio of VACHT to ChAT mRNA is quite different in the head and body of *Drosophila* [7], similar to the situation in peripheral versus central cholinergic neurons in mammals. Various laboratories have inventoried VACHT and ChAT

transcripts of the central and peripheral nervous systems, or cell lines derived therefrom, and the segments of the CGL that are responsible for VACHT and/or ChAT expression in transgenic animals and cell lines (see [3, 18, 20] for reviews). At present, it appears that separate promoter/enhancers may drive VACHT and ChAT expression in a cell-specific manner, while a common exon for VACHT and ChAT (the 'R' exon) suggests that transcription from a single start site generating alternatively spliced VACHT and ChAT transcripts also occurs. Which is the dominant transcriptional mode in mammals? The answer may depend in part on physiological and anatomical context. In the brain, 'R' exon expression is much less than that for VACHT or ChAT orf-containing mRNAs (*figure 2*) This implies that 'R' may be a minor transcriptional pathway, and that separate promoters drive expression of VACHT and ChAT from the CGL, at least under normal physiological conditions in the adult mammalian central nervous

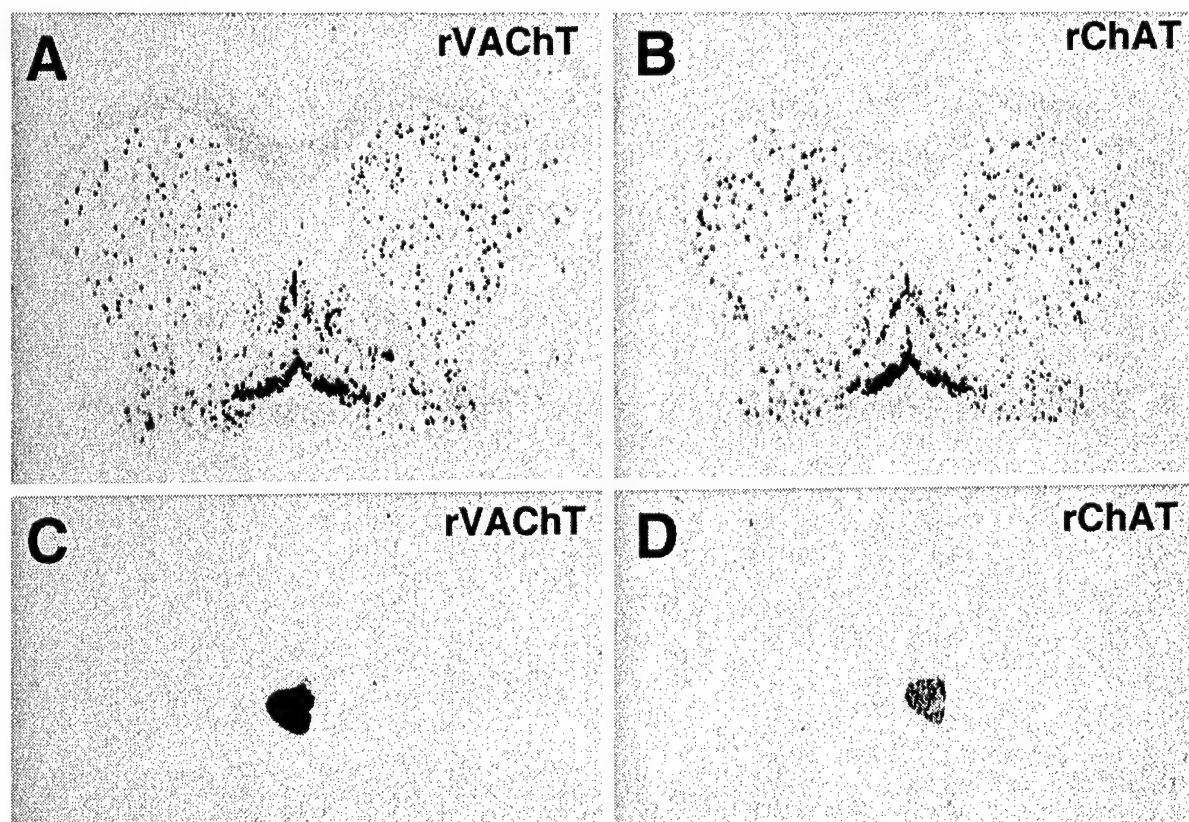


Figure 1. Comparative expression of VACHT and ChAT mRNA in brain and parasympathetic ganglion of adult rat. VACHT (A) and ChAT (B) mRNA levels are similar in adjacent brain sections. Note higher levels of VACHT mRNA (C) as compared to ChAT mRNA (D) in adjacent sections of the otic ganglion. Rat (r) VACHT and rChAT riboprobes of uniform size were synthesized and calibrated for hybridization efficiency as described by Hahm et al. [5].

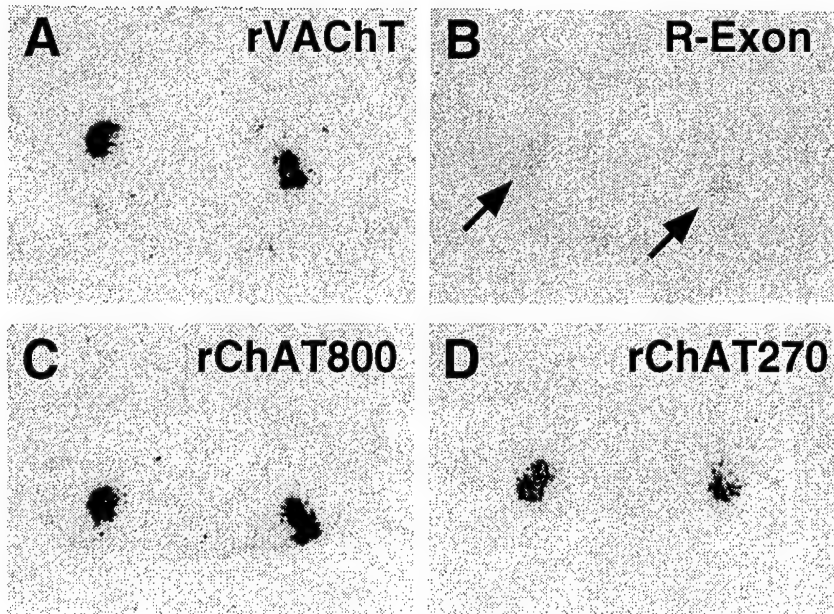


Figure 2. Relative expression of the R, VACHT, and ChAT first coding exon in the rat central nervous system. Sequential sections through rat brain stem hybridized with specific probes for RVACHT orf (A), R-exon (B) and rCHAT orf (C, D). Note the faint hybridization signal for the R-exon. Data from Hahm et al. [5].

system. Consistent with this possibility, Naciff et al. report that a genomic fragment that excludes the R exon and includes only about 600 bases of the CGL upstream of the ATG that initiates the VACHT orf is sufficient for correct cholinergic expression in vivo [8].

The catalytic properties of the VACHT and ChAT proteins themselves suggest that precise regulation of the CGL is required to maintain cholinergic function both centrally and peripherally. The affinity of acetylcholine for the transporter is reckoned to be approximately 1 mM [17, 18], close to the estimated concentration of acetylcholine synthesized in the cytoplasm of motor neurons by ChAT. Thus changes in the ratio of VACHT to ChAT mRNAs, if reflected in differential protein abundance, could directly affect the size of ACh quanta in cholinergic neurons. Increased cholinergic quantal size in *Xenopus* neurons expressing human VACHT support this assertion [14]. VACHT is targeted to small synaptic vesicles (SSVs) both in vivo and in cell lines [4, 19], yet appears to be transported to the nerve terminal via large dense-core vesicles [15]. The vesicular monoamine transporter type 2 (VMAT2) localizes preferentially to large dense-core vesicles (LDCVs) in PC12 cells, and appears to be transported to the nerve terminal in LDCVs as well. Since VMAT2 is localized to both small and large synaptic vesicles in brain [9], PC12 cells apparently produce 'cholinergic' but not 'noradrenergic' SSVs. This finding implies that there are special properties of each type of vesicle besides the mere presence of the appropriate transporter.

Why so many different kind of vesicles? In cholinergic neurons, differential release of ACh and neuropeptides from two different vesicular compartments provides variation in the ratios of ACh and VIP, for example, released at different rates of stimulation (e.g., [1]). Differential targeting to subpopulations of small synaptic vesicles may also be physiologically relevant, for example at developmental stages in which autonomic neurons express both cholinergic and noradrenergic traits [13].

Expression of VACHT and ChAT from the CGL, which makes a neuron primordially cholinergic, also makes the cholinergic neuron amenable to genetic, ontogenic, pathophysiological, and neuroanatomical analysis. Understanding the transcriptional, translational and biochemical regulation of these two proteins ultimately will aid in unlocking the mechanisms by which chemically coded transmission arises during development, serves the needs of the organism, and perhaps goes awry in motor and cognitive neurodegenerative diseases.

Acknowledgments

We wish to acknowledge the collaboration of our laboratories with those of Jim Rand, at the Oklahoma Medical Research Foundation, and Marie-Francoise Diebler, at the Département de Neurochimie, Laboratoire de Neurobiologie Cellulaire, Gif-sur-Yvette, in the original characterization of the mammalian cholinergic gene locus. Supported in part by the German Research Foundation (SFB 397) and Volkswagenstiftung.

References

- [1] Agoston D.V., Conlon J.M., Whittaker V.P., Selective depletion of the acetylcholine and vasoactive intestinal polypeptide of the guinea-pig myenteric plexus by differential mobilization of distinct transmitter pools, *Exp. Brain Res.* 72 (1988) 535–542.
- [2] Alfonso A., Grundahl K., McManus J.R., Asbury J.M., Rand J.B., Alternative splicing leads to two cholinergic proteins in *C. elegans*, *J. Mol. Biol.* 241 (1994) 627–630.
- [3] Eiden L.E., The cholinergic gene locus, *J. Neurochem.* 70 (1998) 2227–2240.
- [4] Gilmor M.L., Nash N.R., Roghani A., Edwards R.H., Yi H., Hersch S.M., Levey A.I., Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles, *J. Neurosci.* 16 (1996) 2179–2190.
- [5] Hahn S.H., Chen L., Patel C., Erickson J., Bonner T.I., Weihe E., Schäfer M.K.-H., Eiden L.E., Upstream sequencing and functional characterization of the human cholinergic gene locus, *J. Mol. Neurosci.* 9 (1997) 223–236.
- [6] Holler T., Berse B., Cermak J.M., Diebler M.-F., Blusztajn J.K., Differences in the developmental expression of the vesicular acetylcholine transporter and choline acetyltransferase in the rat brain, *Neurosci. Lett.* 212 (1996) 107–110.
- [7] Kitamoto T., Wang W., Salvaterra P.M., Structure and organization of the *Drosophila* cholinergic locus, *J. Biol. Chem.* 273 (1998) 2706–2713.
- [8] Naciff J.M., Misawa H., Dedman J.R., Molecular characterization of the mouse vesicular acetylcholine transporter gene, *NeuroReport* 8 (1997) 3467–3473.
- [9] Nirenberg M.J., Chan J., Liu Y., Edwards R.H., Pickel V.M., Ultrastructural localization of the vesicular monoamine transporter-2 in midbrain dopaminergic neurons: Potential sites for somatodendritic storage and release of dopamine, *J. Neurosci.* 16 (1996) 4135–4145.
- [10] Schäfer M.K.-H., Eiden L.E., Weihe E., Cholinergic neurons and terminal fields revealed by immunohistochemistry for VACHT, the vesicular acetylcholine transporter I. Central nervous system, *Neuroscience* 84 (1998) 331–359.
- [11] Schäfer M.K.-H., Eiden L.E., Weihe E., Cholinergic neurons and terminal fields revealed by immunohistochemistry for VACHT, the vesicular acetylcholine transporter II. Peripheral nervous system, *Neuroscience* 84 (1998) 361–376.
- [12] Schäfer M.K.-H., Schütz B., Weihe E., Eiden L.E., Target-independent cholinergic differentiation in the rat sympathetic nervous system, *Proc. Natl. Acad. Sci. USA* 94 (1997) 4149–4154.
- [13] Schütz B., Schäfer M.K.-H., Eiden L.E., Weihe E., Vesicular amine transporter expression and isoform selection in developing brain, peripheral nervous system and gut, *Dev. Brain Res.* 106 (1998) 181–204.
- [14] Song H., Ming G., Fon E., Bellocchio E., Edwards R.H., Poo M., Expression of a putative vesicular acetylcholine transporter facilitates quantal transmitter packaging, *Neuron* 18 (1997) 815–826.
- [15] Tao-Cheng S., Eiden L.E., The vesicular monoamine transporter VMAT2 is targeted to large dense-core vesicles, and the vesicular acetylcholine transporter VACHT to small synaptic vesicles, in PC12 cells, *Adv. Pharmacol.* (1998), in press.
- [16] Usdin T., Eiden L.E., Bonner T.I., Erickson J.D., Molecular biology of vesicular acetylcholine transporters (VACHTs), *TINS* 18 (1995) 218–224.
- [17] Varoqui H., Erickson J.D., Active transport of acetylcholine by the human vesicular acetylcholine transporter, *J. Biol. Chem.* 271 (1996) 27229–27232.
- [18] Varoqui H., Erickson J.D., Vesicular neurotransmitter transporters, *Mol. Neurobiol.* 15 (1997) 165–191.
- [19] Weihe E., Tao-Cheng J.-H., Schäfer M.K.-H., Erickson J.D., Eiden L.E., Visualization of the vesicular acetylcholine transporter in cholinergic nerve terminals and its targeting to a specific population of small synaptic vesicles, *Proc. Natl. Acad. Sci. USA* 93 (1996) 3547–3552.
- [20] Wu D., Hersch L.B., Choline acetyltransferase: celebrating its fiftieth year, *J. Neurochem.* 62 (1994) 1653–1663.

A syntaxin-SNAP 25-VAMP complex is formed without docking of synaptic vesicles

Nicolas Morel^a, Patrice Taubenblatt^a, Monique Synguelakis^b, Gad Shiffa^{a*}

^aLaboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, 91198 Gif-sur-Yvette, France

^bCNRS-UPR 9081, Institut National Agronomique Paris-Grignon, 16, rue Claude-Bernard, 75231 Paris cedex 05, France

Abstract — We show herein that syntaxin is already associated with SNAP 25 and VAMP during fast axonal transport, and in isolated synaptic vesicles, before docking of these secretory organelles at the active zones. (©Elsevier, Paris)

Résumé — Syntaxine, SNAP 25 et VAMP s'associent en absence d'amarrage des vésicules synaptiques à la membrane des terminaisons nerveuses. Nous montrons ici que la syntaxine est déjà associée à la SNAP 25 et la VAMP pendant le transport axonal rapide, et dans des vésicules synaptiques isolées, en absence d'amarrage de ces organites de stockage à la zone active. (©Elsevier, Paris)

axonal transport / SNARE complex / VAMP / SNAP 25

1. Introduction

In motor nerve endings, clusters of synaptic vesicles docked to specialized areas of the presynaptic plasma membrane, the active zones, can be observed [1]. According to the SNARE hypothesis [10], two proteins of the presynaptic membrane, syntaxin and SNAP 25, and a synaptic vesicle protein, VAMP, ensure the specificity of the docking and fusion of the neurotransmitter storing organelles to the presynaptic membrane through the formation of a multiprotein complex. Several cytosolic or membrane proteins will bind sequentially and reversibly to proteins of the SNARE complex during synaptic activity. Using a homogeneous population of cholinergic nerve endings prepared from *Torpedo* electric organ, we showed that syntaxin was recovered in the form of large 16S protein complexes, 65% of SNAP 25 and 15% of VAMP being associated with syntaxin [13].

Synaptic vesicle and presynaptic plasma membrane proteins are synthesized in the neuron cell body and transported to the nerve endings in intra-axonal vesicular membranes. VAMP, syntaxin and SNAP 25 are conveyed by the fast axonal anterograde flow [5, 11]. In order to know if the SNARE complex is assembled only in the nerve endings, we ligated the electric nerves which innervate the electric organs and looked for interactions between syntaxin, SNAP 25 and VAMP which have accumulated upstream from the axonal block. In addition, we looked in purified fractions of isolated cholinergic

synaptic vesicles for the presence of syntaxin and SNAP 25, which participate in the formation of SNARE complex as has been described in brain synaptic vesicles [8].

2. Methods

The anti-syntaxin monoclonal antibodies 10H5 and HPC1 were obtained from Dr M. Takahashi and Sigma Ltd. respectively, anti-SNAP 25 antiserum from Alomone Ltd. (Israel). Anti-VAMP monoclonal antibodies (19K1, 19K2, 19K7) and anti-synaptophysin antiserum were prepared in our laboratory.

Synaptosomes were isolated from *Torpedo marmorata* electric organ as previously described [6]. After hypoosmotic lysis of synaptosomes in buffer A (10 mM Tris buffer, pH 8, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM PMSF), whole synaptosomal membranes were diluted (1 mg protein/mL) in buffer B (100 mM KCl, 10 mM Tris buffer, pH 7.2, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol).

Synaptic vesicles were purified according to Diebler and Lazereg [2]. In ligature experiments, *Torpedoes* were anesthetized (1 g MS 222 (Sandoz) in 3 L sea water). The first electric nerves were ligated where they run around a cartilaginous apophyse. The skin was stitched and the fish kept in sea water at 16 °C. Ligatured nerves were dissected out 48 h later. The fast axonal flow is about four times slower in the case of *Torpedo*, a cold blooded animal, than for mammals. Nerve pieces (1 cm long) were homogenized (100 mg tissue/mL buffer B) in a glass/glass homogenizer. Large debris was removed by a low speed centrifugation (700 g_{max} × 5 min).

Membranes (from synaptosomes or electric nerves) were either directly immunoprecipitated by anti-VAMP magnetic immunobeads (antibody 19K2 bound to goat anti-mouse Ig antibodies linked to M450 Dynabeads) essentially according to manufacturer's protocol (Dyna). Or they were solubilized in buffer B (1 mg protein/mL) by addition of Chaps (30 mM final concentration). In some experiments, membranes (2 mg

*Present address: Howard Hughes Medical Institute, Department of Pharmacology, University of California, San Diego, La Jolla, California 92093-0683, USA

protein/mL in buffer A) were solubilized in SDS (0.5% final concentration) and then 10-fold diluted in 0.5% Triton X-100 in buffer B. Solubilized proteins were recovered in a high speed supernatant and subjected to immunoprecipitation by anti-syntaxin or anti-VAMP immunobeads or to velocity sedimentation through continuous sucrose gradients [11, 12].

After SDS-polyacrylamide gel electrophoresis and electrotransfer, blots were probed with various antibodies, whose binding was indirectly visualized using peroxidase conjugated anti-mouse or rabbit Ig antibodies (Institut Pasteur Production) and the Enhanced ChemiLuminescence system (Amersham).

3. Results and discussion

Recently, we have checked that several presynaptic antigens are transported by the fast axonal flow in *Torpedo* electric nerves and have measured their accumulation upstream from an axonal block [11]. The accumulation in the nerve segment just proximal to the ligature, expressed as percentage of the antigen content in the preceding 1 cm long nerve segment, ranged from five-fold for syntaxin ($480 \pm 170\%$, mean \pm S.E.M. from seven independent experiments) and SNAP-25 ($490 \pm 80\%$, 8 experiments) to 10-fold for synaptotagmin ($1110 \pm 180\%$, nine experiments), VAMP being in between ($665 \pm 5\%$, two experiments). Therefore, syntaxin, SNAP 25 and VAMP in nerve segments proximal to the ligature correspond in majority to antigens transported by the fast anterograde axonal flow in *Torpedo* electric nerves.

In order to know if syntaxin, SNAP 25 and VAMP were transported separately or in association, we performed immunoprecipitation experiments on membrane proteins accumulating proximally to the electric nerve ligature, after solubilization by Chaps or SDS (figure 1). Synaptosomal (S) and nerve membranes proximal to the ligature (P) were solubilized and precipitated in parallel with anti-syntaxin or anti-VAMP immunobeads. An association between syntaxin, SNAP 25 and VAMP was demonstrated. Similar amounts of the syntaxin-SNAP 25-VAMP complexes were recovered, regardless of which detergent was used (either Chaps or SDS). Syntaxin-SNAP 25-VAMP complexes were also immunoprecipitated from solubilized control nerves, in the absence of the ligature (not shown).

The possibility that syntaxin and VAMP associate during solubilization was eliminated through the three following independent experimental arguments [11, 12]. First, similar amounts of syntaxin-SNAP 25-VAMP complexes were recovered both after Chaps or SDS solubilization (figure 1), both from synaptosomes and from ligated nerves. Hayashi and coworkers [3] have shown that the syntaxin-SNAP 25-VAMP complex was SDS-resistant, once formed, but that its constituents were unable to associate if

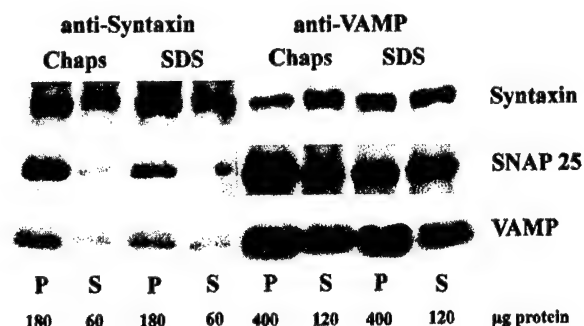


Figure 1. Immunoprecipitation of SNARE complexes solubilized in Chaps or SDS. Membrane proteins from synaptosomes (S) or nerve segments proximal to a ligature (P) were solubilized either by 30 mM Chaps (Chaps), or by 0.5% SDS and then diluted 10-fold in 0.5% Triton X-100 (SDS). Solubilized proteins were immunoprecipitated in parallel on anti-syntaxin (mAb 10H5) or anti-VAMP (mAb 19K1) immunobeads. Syntaxin-SNAP 25-VAMP complexes were detected in both conditions, regardless the detergent used or their synaptosomal or axonal origin.

solubilized in SDS. Second, the amount of syntaxin-VAMP complex was independent of the syntaxin and VAMP concentrations during solubilization of nerve membranes. This complex was recovered even when syntaxin and VAMP concentrations were around 5×10^{-9} M, far below concentrations required to study their association in vitro: EC_{50} in the micromolar range [3, 9]. Finally, using control nerve homogenates where most of syntaxin is not associated with VAMP, we have shown that exogenous VAMP, added at the beginning of the solubilization step, was unable to coprecipitate with syntaxin. Therefore, the axonal syntaxin-SNAP 25-VAMP complexes appear to be formed prior to the solubilization, to be SDS-resistant and extremely stable.

The size and abundance of SNARE complexes in solubilized control or ligated nerve segments were analysed after velocity sedimentation in continuous sucrose gradients (figure 2). Syntaxin distribution, a broad peak around 12-13 S without monomeric syntaxin, was similar in control and ligated nerves. A large proportion of SNAP 25 and VAMP was also recovered in intermediate fractions in ligated nerves, in contrast to control nerves. The syntaxin, SNAP 25 and VAMP peaks were broad, overlapping but not identical, suggesting the existence of several protein complexes containing these antigens. All of these complexes are accumulated in ligated nerves and therefore transported by the fast anterograde axonal flow. In control nerves, most of SNAP 25 and VAMP were recovered in the lightest fractions, probably as mono or dimers. These mono or dimers were also detected but not accu-

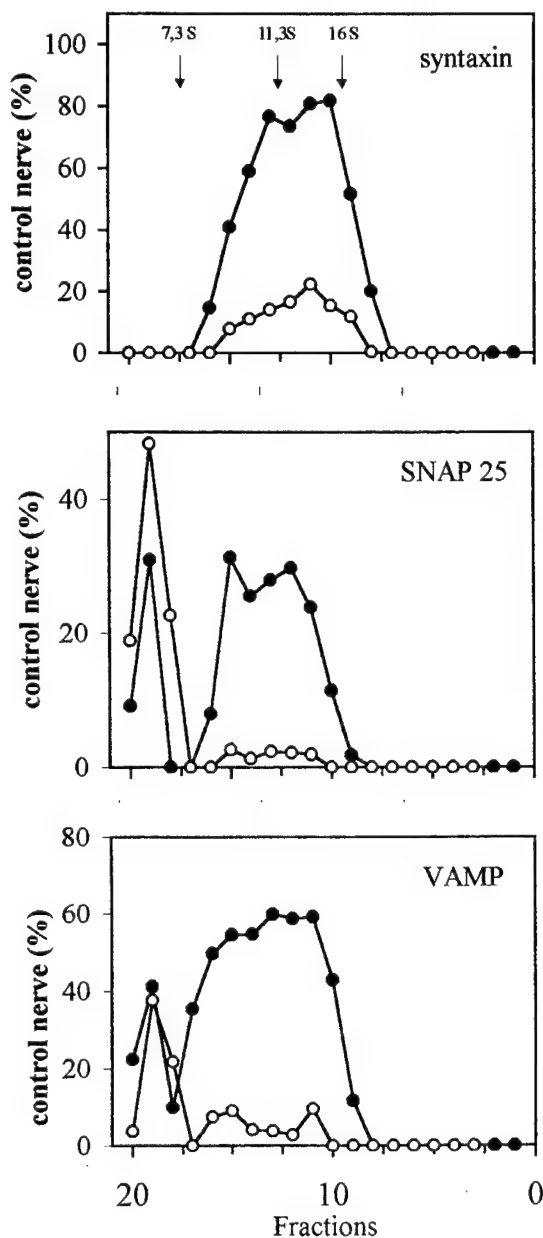


Figure 2. Velocity sedimentation of syntaxin, SNAP 25 and VAMP solubilized from ligated (●) or control (○) nerves. Proteins in electric nerve homogenates (2 mg/mL) were solubilized by 30 mM Chaps and centrifuged (0.4 mL /gradient) in 5-30% linear sucrose gradients in buffer B (40 000 rpm for 18 h in a Beckman SW40 rotor). Fractions were collected from the bottom of the gradients. The syntaxin, SNAP 25 and VAMP contents of each fraction were determined after dialysis and concentration, SDS gel electrophoresis and electrotransfer, and finally immunodetection with specific antibodies (mAb HPC1 and 19K7, anti-SNAP 25 antiserum). Antigen contents are expressed in percentage of the total content of control nerve gradient.

mulated in ligated nerves. In the case of SNAP 25, they correspond to soluble SNAP 25 and are detected in much lower amounts if nerve membranes rather than homogenates are used (not shown).

Purified cholinergic synaptic vesicles are highly enriched in VAMP and synaptophysin, as compared to synaptosomes, but also contain some SNAP 25 (figure 3) and syntaxin (not shown). This confirms previous biochemical [8, 15] and morphological [4] data on brain synaptic vesicles. Using anti syntaxin immunobeads, we have immunoprecipitated syntaxin-SNAP 25-VAMP complexes that are present in the membrane of isolated synaptic vesicles (not shown). It has been suggested that these complexes, involving proteins colocalized in the same synaptic vesicle membrane, are dissociated by NSF and α -SNAP in physiological conditions when cytoplasm is present [8].

If syntaxin, SNAP 25 and VAMP are associated side by side in the same membrane during rapid axonal flow, like in synaptic vesicles, they must be present in the same axonal organelles. Indeed, we have immunoprecipitated VAMP containing nerve vesicles and found that they also contained SNAP 25 (figure 3). Synaptophysin, which is abundant in synaptic vesicles, was not detected in the organelles immuno-isolated from nerves. The present observation should be compared with previous results [7] showing that synaptophysin was transported in organelles that did not contain syntaxin, SNAP 25, nor the vesicular antigen SV2. A better understanding of the sorting of nerve terminal proteins to synaptic vesicles or the presynaptic plasma membrane will require the characterization of the various membrane precursors transported by the fast axonal flow.

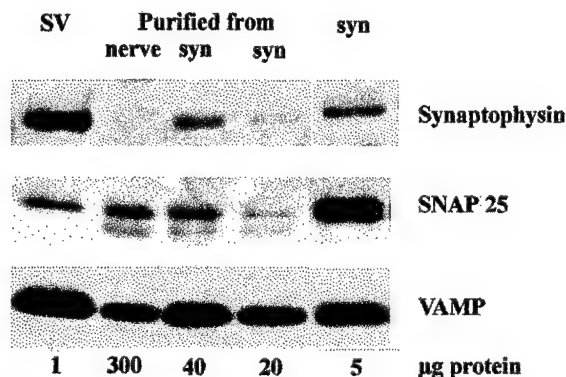


Figure 3. Immunisation of axonal or synaptosomal organelles on anti-VAMP magnetic beads. Vesicle membranes were immunisolated on mAb 19K2 magnetic immunobeads from control nerve homogenates or disrupted synaptosomes. Their synaptophysin, SNAP 25 and VAMP contents were compared to that of purified synaptic vesicles (SV) and of whole synaptosomes (syn).

It was unexpected to find that complexes associating a synaptic vesicle protein, VAMP, and presynaptic membrane proteins, syntaxin and SNAP 25, could have formed outside the active zone, during axonal transport or in isolated synaptic vesicles. In the latter case, Otto and coworkers [8] have shown that these SNARE complexes involved proteins in the same membrane, interacting side by side. This contrasts to the active zone situation where SNARE complexes are believed to be formed by proteins in two opposing membranes. In yeast, side by side SNARE complexes are present in vacuolar membranes and must be dissociated by NSF and α -SNAP to allow homotypic intervacuolar fusion to proceed [14]. The present results suggest that VAMP and SNAP 25 are transported in the same axonal organelles and that axonal SNARE complexes would be similar to those of isolated synaptic vesicles. We still do not know if these SNARE complexes simply form because their three protein constituents are present in the same membranes. Alternatively, they could be required to direct properly presynaptic proteins to the nerve terminal or to avoid premature membrane fusion.

Acknowledgments

This work was supported by AFM grants (to N.M.) and a fellowship to G.S.

References

- [1] Couteaux R.E., Pecot Dechavassine M., Les zones spécialisées des membranes présynaptiques, C.R. Acad. Sci. D. 278 (1974) 291-293.
- [2] Diebler M.F., Lazereg S., Mg-ATPase and *Torpedo* cholinergic synaptic vesicles, J. Neurochem. 44 (1985) 1633-1641.
- [3] Hayashi T., MacMahon H., Yamasaki S., Binz T., Hata Y., Südhof T.C., Nieman H., Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly, EMBO J. 13 (1994) 5051-5061.
- [4] Kretzschmar S., Volkhardt W., Zimmermann H., Colocalization on the same synaptic vesicles of syntaxin and SNAP 25 with synaptic vesicle proteins: a reevaluation of functional models required? Neurosci. Res. 26 (1996) 141-148.
- [5] Li J.-H., Jahn R., Dahlström A., Axonal transport and targeting of the t-SNARE SNAP 25 and syntaxin 1 in the peripheral nervous system, Eur. J. Cell Biol. 70 (1996) 12-22.
- [6] Morel N., Israël M., Manaranche R., Mastour-Frachon P., Isolation of pure cholinergic nerve endings from *Torpedo* electric organ, J. Cell Biol. 75 (1977) 43-45.
- [7] Okada Y., Yamasaki H., Sekine-Aizawa Y., Hirokawa N., The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors, Cell 81 (1995) 769-780.
- [8] Otto H., Hanson P.I., Jahn R., Assembly and disassembly of a ternary complex of synaptobrevin, syntaxin, and SNAP-25 in the membrane of synaptic vesicles, Proc. Natl. Acad. Sci. USA 94 (1997) 6197-6201.
- [9] Pevsner J., Hsu S.-C., Braun J.E.A., Calakos N., Ting A.E., Bennett M.K., Scheller R.H., Specificity and regulation of a synaptic vesicle docking complex, Neuron 13 (1994) 353-361.
- [10] Rothman J.E., Mechanisms of intracellular protein transport, Nature 372 (1994) 55-63.
- [11] Shiff G., Morel N., Association of syntaxin with SNAP 25 and VAMP (synaptobrevin) during axonal transport, J. Neurosci. Res. 48 (1997) 313-323.
- [12] Shiff G., Morel N., Rapid anterograde axonal transport of the syntaxin-SNAP 25-VAMP complex, J. Neurochem. 68 (1997) 1663-1667.
- [13] Shiff G., Synguelakis M., Morel N., Association of syntaxin with SNAP 25 and VAMP (synaptobrevin) in *Torpedo* synaptosomes, Neurochem. Int. 29 (1996) 659-667.
- [14] Ungermann C., Nichols B.J., Pelham H.R., Wickner W., A vacuolar v-t-SNARE complex, the predominant form in vivo and on isolated vacuoles, is disassembled and activated for docking and fusion, J. Cell Biol. 140 (1998) 61-69.
- [15] Walch-Solimena C., Blasi J., Edelman L., Chapman E.R., Fischer von Mollard G., Jahn R., The t-SNARE syntaxin 1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling, J. Cell Biol. 128 (1995) 637-645.

Conclusions and comments. Xth International Symposium on Cholinergic Mechanisms

Alexander G. Karczmar

*Research Services, 151, Hines VA Hospital, Hines, Illinois 60141, USA, Department of Pharmacology and Experimental Therapeutics
and Loyola University Medical Center, Maywood, Illinois 60153, USA*

Abstract — Ancient medicine men of Egypt and Arabia employed, under another name, the cholinergic agents, as did the hunters, warriors and shamans of Africa and South America. An explosion of cholinergic science occurred in the last and the current century, and the ISCMs witnessed and catalyzed this progress. The Xth ISCM emphasized the molecular characteristics of the receptors, cholinesterase and of the system engaged in liberation of Ach. (©Elsevier, Paris)

Résumé — Conclusions et commentaire. X^{ème} Symposium International sur les Mécanismes Cholinergiques. L'emploi des agents cholinergiques est ancien; il a commencé avec les guérisseurs Arabes et Egyptiens et les chasseurs, guerriers et shamans d'Afrique et d'Amérique du Sud. Le dernier siècle, ainsi que le nôtre, ont vu une explosion de la science cholinergique, dont les SICMs ont été les témoins et les catalyseurs. Le focus du X^{ème} SICM a été sur les caractéristiques moléculaires de récepteurs, de la cholinésterase et de la libération d'ACh. (©Elsevier, Paris)

cholinergic / central nervous system / Alzheimer's disease / transmission

1. Introduction

The progress and the success of the cholinergic field is, arguably, beyond that of any other field of biological knowledge. The field was named by Sir Henry Dale, but it antedates Dale by millennia, as it begins with the BC Egyptian and Arab medicine. The Calabar natives and the South American Indians followed with the hunt, rite and war use of curari-mimetics and their antidotes [12]. And then, there was an outburst of modern studies which begun with the work of Scotch missionaries and Edinburgh pharmacognosists and doctors of the 1850s; then, there were the studies of German, French, English and American pharmacologists which lead to the discovery of the chemical transmission, at the periphery in the nineteen twenties and in the central nervous system 30 years later [18].

Since then, the past nine International Symposia on Cholinergic Mechanisms (ISCMs) initiated by Edith Heilbronn were the witnesses of and contributed to, the further, exponential progress of the cholinergic lore. Thus, the Ist 1970 ICSM presented data on biosynthesis and chemical analysis of acetylcholine (ACh), three or so muscarinic receptors and a few behavioral correlates of the cholinergic system, all departures from what was known in the 1960s, while the IXth, Mainz ISCM confronted us with many more muscarinic and nicotinic receptors, second and third messengers characteristics of their activation, molecular biology of the components of the cholinergic system, trophic factors and cholinergic corre-

lates of dementia [21, 25]. And, we saw for ourselves, that the Xth ISCM was not a poor relative of the preceding ISCMs.

Of course, the early giants of cholinergicity, Dale, Loewi, Hunt, Dixon, Bovet, Koppanyi, Nachmansohn, MacIntosh passed away; the memory of Nachmansohn and of the Arcachon laboratories where he was so active were vividly depicted at this Symposium by Jean-Pierre Changeux and Victor Whittaker. I regret the absence of Sir William Feldberg, Bo Holmstedt, Israel Hanin, Peter Waser and Don Jenden at this Symposium. It is sad that the progress made since the last ISCM had to be marred by the passing away of their two worthy descendants, George Koelle and Sir John Eccles. George, who participated in several of ISCMs, was honored at this Symposium by Dr. Jack MacMahan. It was not possible to acknowledge similarly the more recent departure of Jack Eccles (May 2, 1997), and these few lines must suffice at present.

Sir John C. Eccles did for the central nervous system what Otto Loewi did for the peripheral nervous system: he demonstrated, via his Renshaw cell experiments [5, 6], the central presence of a chemical, cholinergic system, experiments that were responsible in part for his Nobel Prize award. But Eccles did much more than that, as he described, with Ben Libet and Rosamond Eccles, the muscarinic and nicotinic potentials of the sympathetic ganglia; explained, with Andrew Huxley and Alan Hodgkin, the ionic mechanisms of excitatory and inhibitory potentials; defined the circuitry, transmissive charac-

mann, Changeux, Daniel Bertrand and Edson Albuquerque contributed to the study of the diversity of nicotinic receptors, their molecular biology and function, while Sir Arnold Burgen, Hammer, Heller-Brown, Hartzell, Dowdall, Ladinsky, Schimerlik, Bob Schwartz, and Ed Hulme focused similarly on the muscarinic receptors.

It is accepted today (see, for example, Galzi and Changeux [7] and Hucho and Tsetlin [13]) that the nicotinic receptors (NACHRS) are allosteric glycoproteins that are structured into pentameric combinations of alpha and beta subunits surrounding the cation-selective channel; in view of this allostericity these receptors may exist in several conformations. As shown at this Symposium, this organization is characterized by wide flexibility and lends itself to mutational and functional changes and to the generation of many heterologous and homo-oligomeric forms of the receptor. Thus, John Dani demonstrated that the $\alpha 7$ subunit plays the transmissive role as it mediates Ca^{2+} influx, Daniel Bertrand showed the importance of $\alpha 4$ and $\alpha 7$, and $\beta 2$ oligomeric receptors for the NACHR sensitivity for agonists and desensitization processes (see also Corringer et al. [3]), Hucho and his collaborators defined the receptor conformation that exhibits high affinity/closed channel state and described the amino acid character of the accessible extramembrane of the receptor, while Steven Sine (see Watty et al. [36]) presented data indicating that the peptide loops that contribute to the non-alpha subunits regulate the agonist and antagonist affinities of the nicotinic receptors. Looking at the other side of the story Andre Menez investigated the cysteine spacing and the structural characteristics of toxins that explain their 'folding' properties (see also Drakopoulou et al. [4]) and thus may clarify certain ligand-binding properties of the NACHR. Marc Ballivet established a new family of NACHRs exhibiting a novel assembly $\alpha 3$ and $\alpha 5$ and β -subunits and described the significance of these and other subunits in the generation of currents of various conductance characteristics and in NACHR capacity for rapid desensitisation. In a different vein, he defined neurogenetic and evolutionary significance of genes controlling transcription factors of the helix-loop-helix proteins and of α -subunits (see also Roztocil et al. [34]).

The desensitization data presented by Marc Ballivet and Daniel Bertrand pertain to the story that begun with Steve Thesleff, Paul Greenagard and Edson Albuquerque [2]; it should be added that another allosteric change in the nicotinic receptor leads to an opposite process, that of sensitization [29], and I did not hear much about this particular phenomenon at the ISCMs!

Jean Cartaud, Fabrizia Bignami and their associates focused on the important area concerning relations between extrinsic and intrinsic proteins and the neuronal organelles. Their results indicate that in the *Torpedo* the intrinsic protein, NACHR communicates with the extrinsic protein, rapsyn, a NACHR clustering agent, via targeting intracellular organelles, such as those present in the Golgi apparatus or microtubules; this system is a part of the secretory, ACh release, pathway. Other, axon derived proteins, such as agrin and neuroregulin, and those derived from muscle, such as laminin 11 participate in the differentiation of the postsynaptic membrane, nerve terminals and terminal Schwann cells of the neuromyal junction, as explained by Josh Sanes and Steve Burden.

Continuing his long-time work on muscle disease, Andrew Engel concentrated on the genetic character of the myopathies resulting from deficiency of the lysosomal acid α -glucose (glycogen storage diseases); furthermore, Engel characterized the receptor modifications and the resulting transmission change in several forms of myasthenia gravis.

Now, as to the muscarinic receptors (MACHRs). Juergen Wess and his collaborators suggested that multiple intracellular receptor loops or domains within the α -subunits regulate the specificity of the G protein links with several, including the muscarinic receptors. Ed Hulme, continuing his work on the transmembrane helices of M receptors [14] found that within the α -helix of the domain 3 of the M1 receptor a specific band of amino acids that include an aspartate-arginine-tyrosine triad is critical for the G protein links as well as for 'efficient' receptor folding and thus for agonist and antagonist affinities.

As to the developmental aspects of the MACHRs Neil Nathanson demonstrated the significance of the GATA family of transcription factors for transactivation of the cardiac cm2 receptor gene in the chick; he also discussed the chick retinal trophic (secretory) factor that increases selectively expression of some MACHR receptors but not of others. Of special interest is his finding that certain trophic and related factors that were previously linked with the regulation of choline acetyltransferase transcription also regulate the transcription of the muscarinic receptor gene (see also Nathanson [31]).

2.3. Cholinesterases

This family of enzymes constitutes another, important component of the cholinergic transmission. Recent work of Jean Massoulie, Israel Silman and Hermona Soreq (see Heilbronn [10]) yielded an image of AChE as having a 'gorge' on the esteric

site and a flatter anionic site; their work also defined amino acid composition of these sites and the multitude of molecular and physical forms that AChE may assume.

At this Symposium, Mona Soreq and her associates continued their life-long investigations of the genetics, the structure, and the function of AChE. Using transgenic and antisense models particularly concerning neurexin gene family-4, they described genetic, functional and circuitual regulation of the generation of AChE variants. They also stressed their neuronal trophic effects, an effect described early by George Koelle, and antitrophic action of their overexpression.

The capacity of AChE to assume various physical and structural forms was emphasized by several investigators. Thus, Jean Massoulie described the genetics and the expression of physical forms of AChEs that relate to congenital conditions such as myasthenia and to deficits of junctional AChE, Dick Rotundo defined the developmental mechanisms involved in organization and structurization of AChE of the endplate, while Israel Silman referred to various folding and unfolding states of AChE that exhibit several 'molten molecule' states and hydrophobic cores. He also defined the structures of several OP-AChE conjugates and of their dealkylation products that result in 'aging' or non-reactivability; he speculated that the junctional membrane participates, via energy mechanisms, in insertion and translocation of these various AChE states and in their affinity regulation. Finally, Palmer Taylor employed crystalline forms of mouse and *Torpedo* AChE and peptide toxins to relate AChE templates to ligand, OP and toxin binding, as well as to the route of substrate entry and its contact points with AChE. He stressed particularly the importance of the dimensions and composition of the acyl pocket, the choline binding site, and of the vicinity of the active center gorge.

2.4. Functions and behaviors

The role of receptors is to elicit, beyond their synaptic function, organ activity and behavior. Steve Arneric and his associates showed that nicotinic agonists of 3-pyridyl ether series attenuate, in several species, pain evoked by a number of pain stimuli and neuropathic pain states; they demonstrated that these compounds do not induce dependence in animals. These are important findings in view of the well known – disputed by very few pharmacologists such as David Warburton – addictive action of nicotine. It should be added that both nicotinic and muscarinic substances, as well as anti-ChEs are analgetic, and that this effect may be – there is a con-

troversy here, see Karczmar [19] – non-opioid in nature. As a counterpart of Arneric's presentation, Emilio Merlo-Pich, who previously studied ethanol addiction (see Koob et al. [30]), defined the genetics and the neural pathways regulating the nicotinic addiction as he found that reward-related activation of central nicotinic receptors via nicotine self-administration by 'addicted' rats results in c-fos expression in mesocorticolimbic dopaminergic system. As to other behavioral effects, Mohammed Shoaib and his associates hypothesized a cholinergic-glutamatergic interaction as they showed that certain NMDA receptor antagonists prevent the up-regulation of nicotinic receptors upon chronic nicotine administration and antagonize the resulting increase in nicotine locomotor effect, while locomotor, learning and other dysfunctions were described by Steve Heinemann, a pioneer in the studies of molecular and functional aspects of NACHRs for mice which suffered a 'knock-out' of their $\alpha 9$ -nicotinic subunit.

These presentations refer to a few, important behavioral aspects of the NACHRs. Yet, it must be remembered that there is no known behavior that does not exhibit cholinergic correlates, and that these include the muscarinic as well as the nicotinic system (see Karczmar [19, 21, 23]). Steve Heinemann expressed hope that molecular neurobiology will reveal the genetics of these behaviors as well as of many disease states.

2.5. Cholinergic transmission

The classical notion elaborated particularly by Eduardo di Robertis and Victor Whittaker was that cholinergic transmission depends, via specialized presynaptic elements, on vesicular release of ACh and its direct action on postsynaptic or postjunctional receptors. Important new understanding of this early model concerns the vesamicol-sensitive protein controlling the uptake of the synaptically released ACh, the transport proteins regulating the movement, fusion with the presynaptic membrane and recycling of the vesicles, and the protein regulators of the release of ACh [9, 15]. At this Symposium, Louis Hersh and Carlos Ibanez-Moliner discussed the cholinergic gene locus which comprises genes for choline acetyltransferase (ChAT) and for vesicular ACh transporter (VACHT), as they demonstrated the role of protein kinase A II and other molecular mechanisms in regulation of the expression of both these genes. Jacques Mallet, Heinrich Betz and Jeffrey Erickson identified the molecular mechanisms and the domains which regulate, in a structure/function relationship, ACh transport, targeting of the synaptic vesicles, and vesicular fusion; in this context, Jeffrey

Erickson developed a new assay for the analysis of the kinetics of the VACHT.

A revolutionary attempt at changing the classical view was made by Israel, Dunant and Descarries. Maurice Israel and Yves Dunant employed ultra-rapid, ultra-sensitive method for ACh measurement to describe in the 1980s a non-vesicular, cytoplasmic release of ACh (pace Victor!) that was regulated by a proteolipid, mediatoaphore, and which actually, may be, as described by Israel at this Symposium, quantal in nature. Then, Laurent Descarries demonstrated, first for the serotonergic and noradrenergic and then for the cholinergic transmission that the classical synaptic differentiation may be, in several brain parts, a *rara avis*! Thus, besides synaptic, 'diffused' transmission may be obtained. He feels that the presence of extra-synaptic NACHRs and MACHRs and special species of AChE, such as the globular G4 form at these sites, and, particularly, persistent level of ACh in the extracellular spaces in several brain regions, all advocate the existence of diffuse transmission. As indicated by the ardent discussion at this Symposium of the views of Descarries, Dunant and Israel, the matter is still a subject of controversy!

2.6. Alzheimer's disease (AD) and its treatment

Involvement of the cholinergic system in and the facilitatory effect of cholinergic agonists on, memory and learning is known since the nineteen forties (see Karczmar [17]). Thus, when the Perrys and the McGeers and Whitehead (see Thal [35] and Giacobini [8]) demonstrated that significant damage to cholinergic neurons occurs in AD, the field was open for cholinergic therapy of AD. In the last 10 years, Marsel Mesulam confirmed and expanded our understanding of this damage in AD patients, and he continued defining it at this Symposium. While it is known that NACHRs are affected in AD, Mesulam explored further changes in the MACHRs. He demonstrated a decrease in the m2 presynaptic autoreceptors located on the cholinergic radiation originating in the nucleus basalis of Meynert as well as a diminution of the frequency of the m2 cortical postsynaptic receptors; as the latter express NO synthetase, Mesulam speculates that their loss contributes to the loss of cortical plasticity; he discussed also the important and controversial problem of the relation between the changes in the cholinergic system and the beta amyloid metabolism. It must be added that there is a controversy as to both qualitative and quantitative changes of other MACHR subtypes in AD. As stressed by Mesulam, the neuronal damage extends in AD beyond the cholinergic neurons. Ezio Giacobini was particularly active, after the early di-

sappointments with the physostigmine treatment of AD, in the development of antiChEs that would penetrate well into the CNS, induce a protracted inhibition of AChE, possibly exert selective effects at strategic sites and receptors and, of course, cause minimal side effects, and he presented at this Symposium the current data on this matter.

Today, AD appears to be a complex, genetically controlled apoptotic process that involves the formation of abnormal proteins (β -amyloids, τ -proteins) and an interaction between such factors as presenilins, proteases, protein precursors and, possibly, active radicals (see Karczmar [27]). This general scheme was further analyzed at this Symposium. Danny Michaelson emphasized that the AD pathognomy includes an apolipoprotein E mutant in which the hyperphosphorylation of the microtubular tau protein proceeds unimpeded and he described a loss of cholinergic as well as serotonergic and adrenergic neurons and memory and learning deficits in congenital apolipoprotein E-deficient mice. Chris Henderson suggested that the loss of trophic factors may also be a component of AD. And Steven Younkin demonstrated the importance in the early-onset AD type of mutations in presenilin 1 (PS1) gene on chromosome 14 as they lead to elevation of highly amyloidogenic peptides; he also defined the gene locus of the most effective mutation.

Altogether, AD cannot be considered as a cholinergic disease; there are also other reasons for expecting only a limited success with antiChE or cholinergic agonist therapy of AD [8, 22]. Indeed, even the newest, FDA, USA-approved drug, donepezil (Aricept), while ameliorating scores of certain subtle cognitive tests, does not improve the quality of life and restore the independence of the AD patients [33]. However, Danny Michaelson found that in his apolipoprotein E deficient mouse model a synthetic MACHR-1 agonist abolished their working memory impairments.

Altogether, non-cholinergic treatments are of importance. Chris Henderson described a synergistic trophic action on the motoneurons of a glial factor and of cardiotrophin-1, as a model for such a treatment, while Anders Bjorklund demonstrated in rats facilitatory cognitive effects and prevention of learning deficits in the course of aging when NGF was administered to middle-aged rats in the form of transplants of NGF-secreting neural progenitor cells. It must be added that at the VIIIth and IXth ISCMs of Claudio Cuello and Konrad Loeffelholz additional, potentially useful in AD trophics were described. Other alternative treatments are being tested at this time, including hormonal and anti-inflammatory therapies [28].

2.7. Toxicological threat of anticholinesterases

Anti-ChEs are highly toxic agents, as certain OP agents kill experimental animals at fraction of $\mu\text{g/kg}$ doses, and as a few cc of their vapors may be deadly to hundreds of exposed people. In man, the anti-ChE death is due primarily to the prolonged depolarization and then desensitization of the cholinceptive neurons of the respiratory centers and, subsequently, to the paralysis of the respiratory muscles. In some subjects and in some animal species the death may be muscarinic and 'asthmatic' in nature [20].

In the 1930s, German investigators and their government became aware of this toxicity and of the war potential of OP anti-ChE nerve agents and conducted vigorous research concerning their development, use as nerve agents, and antidoting. Inevitably, this was followed by similar efforts in the UK, France, USA and USSR (see Holmstedt [11] and Karczmar [28]). This research continues, and Dave Moore presented at this Symposium effects of subtoxic doses of nerve agents in human volunteers and in animals.

While classically the toxicity of OP and other anti-ChEs relates to the inhibition of ChEs and accumulation of ACh, Dave Moore's studies concern certain non-classical aspects of anti-ChE toxicity. These include 'ginger-Jake' paralysis caused at the end of the XIXth Century by tetra-o-cresyl phosphate and described since that time for other OP agents as the organophosphorus ester-induced neurotoxicity (OPIDN), and the delayed cognitive toxicity (DCT), defined first by F. H. Duffy and J. L. Burchfiel (see Karczmar [28]). Actually, that OP drugs may cause neuronal damage and myopathy has been recognized for the last 20 years [20, 23]. The mechanism of this damage is not clear; in the case of the neuronal OPIDN it may be related either to inhibition of a somewhat hypothetical enzyme, the 'neurotoxic esterase' [16] or to neuronal hyperactivity [1]. Wolf Dettbarn, one of the brilliant past associates of Dave Nachmansohn who focused for many years on the mechanisms of OP-induced myopathy, presented at this Symposium convincing evidence concerning the contribution of the hyperactivity and the peroxidation processes to this pathology.

While these agents were not used in the Second World War, there was documented accidental toxicity in relation with their manufacture. Furthermore, they may have been employed subsequently by Iraq and in Yugoslavia; many rogue states are capable of their development, and there is a record of their use by terrorists, as described at this Symposium by Kazuhito Yokoyama with regard to the Sarin attack in the Tokyo subway. Exposure to these agents during

the Persian Gulf War of 1990/1991 relates to the Persian Gulf War Syndrome (PGWS). The PGWS is an unfinished matter; it is controversial within the USA, as well as between the official USA sources and certain French and Czech investigators, as referred to at this Symposium by Guy Blanchet (see also Karczmar [21]).

Besides DCT, OPIDN and other pathological actions of OP drugs, other novel factors may contribute to the PGWS. Thus, at this Symposium, Alon Friedman and Mona Soreq suggested that stress may damage the blood-brain barrier and facilitate brain penetration of drugs such as pyridostigmine that are included in the drug 'cocktail' employed to prevent or antagonize OP toxicity. This finding expands on the 1940s and 1950s demonstration that anti-ChEs facilitate penetration into the CNS of a number of pharmacological agents [17].

Altogether, pyridostigmine is not an innocent member of the cocktail. Its neurotoxicity was described at this Symposium by Peter Spencer, while Edson Albuquerque and his associates demonstrated recently direct channel effects of very low doses of pyridostigmine and OP drugs that may contribute to their AChE inhibition-related toxicity; they also stressed, at this Symposium, the role of nicotinic receptors and channels, and of endogenous choline, in the regulation of behavior and cholinergic toxicity.

3. Envoi

This Xth ISCM was a testimonial to the progress of cholinergic lore as well as a further contribution to its status. It focused on the molecular aspects of the cholinergic system, as it outlined the genetic mechanisms underlying its components, their variety and neurogenesis, and as it described the various NACHRs and the MACHRs, ChEs, ChAT and the proteins regulating transport and release of ACh, whether vesicular, cytoplasmic or diffuse. The power of the methodology undergirding this progress and the elegance of our present understanding of these items is nothing short of astounding. Thus, the Xth ISCM leaves us with an image of molecular interaction between the subunits of the receptors and ChEs, and between the receptors and the regulatory extrinsic and intrinsic proteins and organelles which, while complex, makes functional sense and underlies the flexibility and plasticity of the cholinergic system.

If I may gripe, it would be only with regard to the emphasis of this Symposium on the microstate of this knowledge which does not directly translate into the macrostate, such as the overt behaviors. A few references were made to the behaviors that are

endowed with cholinergic correlates such as pain, attention and addiction, but a concerted effort at presenting the general significance of the multiplicity of cholinergic behaviors was not made at this or other recent ISCMs. Such significance was hinted at by Stephen Arneric as he stressed that the stimulation of NACHRs reduces the negative effect of distraction on learning (see also Prendergast et al. [32]). This demonstration adds support to the notion that cholinergic, both nicotinic and muscarinic, agonists induce a specific behavioral syndrome, the cholinergic alert non-mobile behavior (CANMB) as they promote organism-environment interaction [24].

It must be stressed that the exploitation of the cholinergic system represents not only a cornucopia of plenty, but also a Pandora's box of calamities, and this aspect of the matter was presented as well, as we dwelled on the use of the OP agents by terrorists and rogue states. An additional example of this mixed blessing status of cholinergic agents concerns the agricultural use of anti-ChEs as insecticides and pesticides. This use constitutes a difference between life and death for undeveloped nations, yet each year there are, worldwide, thousands of fatalities related to this use [21] – a matter that has not been not discussed at this or other ISCMs. Altogether, the exploitation of the cholinergic system is of immense benefit to mankind as well as of potential danger.

Acknowledgments

This author's research referred to in this paper was supported in part by the NIH grants NS06455 and GM77, CARES of Chicago, Hines VA Hospital Research Service and AMVETS of Illinois.

References

- [1] Abou-Donia M.B., Lapadula D.M., Mechanism of organophosphorus ester-induced neurotoxicity: Type I and Type II, *Annu. Rev. Pharmacol. Toxicol.* 30, 405-440.
- [2] Albuquerque E.X., Allen C.N., Aracava Y., Akaike A., Shaw K.P., Rickett D.L., Activation and inhibition of the nicotinic receptor: actions of physostigmine, pyridostigmine and meprodifen, in: Hanin I., (Ed.), *Dynamics of Cholinergic Function*, Plenum Press, New York, pp. 677-695.
- [3] Corringer P.J., Bertrand S., Edelstein S.J., Changeux J.-P., Bertrand D., Critical elements determining diversity in agonist binding and desensitization of neuronal acetylcholine receptors, *J. Neurosci.* 18 (1998) 648-657.
- [4] Drakopoulou E., Vizzavona J., Neyton J., Aniot V., Bouet F., Virelizier H., Menez A., Vita C., Consequences of the removal of evolutionary conserved disulfide bridges on the structure and function of charybotoxin and evidence that particular cysteine spacings govern specific disulfide bond formation, *Biochemistry* 37 (1998) 1292-101.
- [5] Eccles J.C., Katz B., Koketsu K., Cholinergic and inhibitory synapses in a central nervous pathway, *Aust. J. Sci.* 16 (1953) 50-54.
- [6] Eccles J.C., Katz B., Koketsu K., Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurons, *J. Physiol. (Lond.)* 126 (1954) 524-562.
- [7] Galzi J.-L., Changeux J.-P., Neuronal nicotinic receptors: Molecular organization and regulation, *Neuropharmacology* 34 (1995) 563-582.
- [8] Giacobini E., New trends in cholinergic therapy for Alzheimer disease; nicotinic inhibitors or cholinesterase inhibitors?, in: Klein J., Loeffelholz K. (Eds.), *Cholinergic Mechanisms: From Molecular Biology to Clinical Significance*, Elsevier, Amsterdam, 1996, pp. 311-323.
- [9] Greengard P., Neuronal phosphoproteins, *Neurobiology* 1 (1987) 81-119.
- [10] Heilbronn E., Molecular biology of cholinesterases: a background and an introduction, in: Cuervo A.C. (Ed.), *Cholinergic Function and Dysfunction*, Elsevier, Amsterdam, 1993, pp. 133-138.
- [11] Holmstedt B., Structure-activity relationship of the organophosphorus anticholinesterase agents, in: Koelle G.B. (Ed.), *Cholinesterases and Anticholinesterase Agents*, Springer-Verlag, Berlin, 1963, pp. 428-485.
- [12] Holmstedt B., The ordeal bean of old calabar. The pageant of *Physostigma venenosum* in medicine, in: Swain T. (Ed.), *Plants in the Development of Modern Medicine*, Cambridge University Press, Cambridge, 1972, pp. 303-360.
- [13] Hucho F., Tsetlin V., Structural biology of key nervous system proteins, *J. Neurochem.* 66 (1996) 1781-1792.
- [14] Hulme E.C., Birdsall N.J.M., Buckley N.J., Muscarinic receptor subtypes, *Annu. Rev. Pharmacol. Toxicol.* 30 (1990) 633-673.
- [15] Israel M., Morel N., Mediatophore: A nerve terminal membrane protein supporting the final step of acetylcholine release process, in: Aquilonius S.-M., Gillberg P.-G. (Eds.), *Cholinergic Neurotransmission: Functional and Clinical Aspects*, Elsevier, Amsterdam, 1990, pp. 101-110.
- [16] Johnson M.K., The target for initiation of delayed neurotoxicity by organophosphorus ester: Biochemical studies and toxicological applications, *Rev. Biochem. Toxicol.* 4 (1982) 141-202.
- [17] Karczmar A.G., Pharmacologic, toxicologic and therapeutic properties of anticholinesterase agents, in: Root W.S., Hoffman F.C. (Eds.), *Physiological Pharmacology*, Academic Press, New York, 1967, pp. 163-322.
- [18] Karczmar A.G., History of the research with anticholinesterase agents, in: Karczmar A.G. (Ed.), *Anticholinesterase Agents*, vol. 1, Section 13, *Int. Encyclop. Pharmacol. Ther.*, Pergamon Press, Oxford, 1970, pp. 1-44.
- [19] Karczmar A.G., Central actions of acetylcholine, cholinomimetics and related drugs, in: Goldberg A.M., Hanin I., (Eds.), *Biology of Cholinergic Function*, Raven Press, New York, 1976, pp. 395-449.
- [20] Karczmar A.G., Acute and long lasting central actions of organophosphorus agents, *Fund. Appl. Toxicol.* 4 (1984) 1-17.
- [21] Karczmar A.G., Physiological cholinergic functions in the CNS, in: Aquilonius S.-M., Gillberg P.-G. (Eds.), *Cholinergic Neurotransmission: Functional and Clinical Aspects*, Elsevier, Amsterdam, 1990, pp. 436-466.
- [22] Karczmar A.G., SDAT models and their dynamics, in: Becker R., Giacobini E. (Eds.), *Cholinergic Basis for Alzheimer Therapy*, Birkhauser, Boston, 1991, pp. 141-152.

- [23] Karczmar A.G., Brief presentation of the story and present status of studies of the vertebrate cholinergic system, *Neuropsychopharmacology* 9 (1993) 181–199.
- [24] Karczmar A.G., Cholinergic substrates of cognition and organism-environment interaction, *Prog. Neuro-Psychopharmacol. Biol. Psychiat.* 19 (1995) 187–211.
- [25] Karczmar A.G., Loewi's discovery and the XXI Century, in: Klein, J., Loeffelholz K. (Eds.), *Cholinergic Mechanisms: From Molecular Biology to Clinical Significance*, Elsevier, Amsterdam, 1996, pp. 1–27.
- [26] Karczmar A.G., Sir John Eccles: A testimonial, *Persp. Biol. Med.*, (1998), in press.
- [27] Karczmar A.G., Anticholinesterases: dramatic aspects of their use and misuse. *Neurochem. Int.* (1998), in press.
- [28] Karczmar A.G., Crayton J., Siegel G., Novel combined treatment for Alzheimer's disease and stroke. Abstracts, Xth International Symposium on Cholinergic Mechanisms, (1998), in press.
- [29] Koketsu K., Karczmar A.G., Action of NaF at various cholinergic synapses, *Fed. Proc.* 25 (1966) 627.
- [30] Koob G.F., Roberts A.J., Schulties G., Parsons L.H., Heyser C.J., Hyytia P., Merlo-Pich E., Weiss F., Neurocircuitry targets in ethanol reward and dependence, *Alcohol Clin. Exp. Res.* 22 (1998) 3–9.
- [31] Nathanson D.M., Differential requirements for p21ras and protein kinase C in the regulation of neuronal gene expression by nerve growth factor and neurokinins, *J. Biol. Chem.* 269 (1994) 18856–18863.
- [32] Prendergast M.A., Jackson W.J., Terry A.V. Jr., Decker M.W., Arneric S.P., Buccafusco J.J., Central nicotinic receptor agonists ABT-418, ABT-089, and (-)-nicotine reduce distractibility in adult monkeys, *Psychopharmacol.* 136 (1998) 50–58.
- [33] Rogers S.L., Farlow M.R., Doody R.S., Mohs R., Friedhoff L.T., the Donepezil Study Group, A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease, *Neurology* 50 (1998) 136–151.
- [34] Roztocil T., Matter-Sadzinski L., Alliod C., Ballivet M., Matter J.M., NeuroM, a neural helix-loop-helix transcription factor, defines a new transition stage in neurogenesis, *Development* 124 (1997) 3263–3272.
- [35] Thal L.J., Clinical trials in Alzheimer disease, in: Terry R.D., Katzman R., Bick K.L. (Eds.), *Alzheimer Disease*, Raven Press, New York, 1994, pp. 431–444.
- [36] Watty A., Methfessel C., Hucho F., Fixation of allosteric states of the nicotinic acetylcholine receptor by chemical cross-linking, *Proc. Natl. Acad. Sci. USA* 94 (1997) 8202–8297.

Contribution of nAChRs to the function of CNS synapses: The action of choline as a selective agonist

E.X. Albuquerque, M. Alkondon, E.F.R. Pereira.

Dept. Pharmacol. Exp. Ther., Univ. Maryland Sch. Med., Baltimore, MD 21201, USA

It is generally acknowledged that the hippocampus, an area of the central nervous system (CNS) that is involved in processing cognitive functions [10], is very sensitive to cholinergic modulation, and that the density of neuronal nicotinic acetylcholine receptors (nAChRs) in the hippocampus is severely diminished in diseases characterized by learning and memory impairment (e.g., Alzheimer's disease) [12]. Thus, the developing hippocampus has become the focus of our interest with regard to characterization of the nAChR function. One of the major hindrances to addressing nAChR function in the brain was the lack of specific agonists and antagonists for each of the receptor subtypes. This problem was further aggravated by the fast kinetics of inactivation of some of the neuronal nicotinic receptors, particularly of those composed of the $\alpha 7$ subunit. The field has now advanced immensely, and many of these problems have been overcome. Our initial work in this field demonstrated for the first time that cultured hippocampal neurons respond to nicotinic agonists with at least one of three types of whole-cell currents referred to as IA, II, and III [1]. These currents, which can be distinguished from one another on the basis of their kinetic and pharmacological properties, are subserved by $\alpha 7$, $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChRs. Type IA currents, by far the predominant response of hippocampal neurons to nicotinic agonists, are fast-desensitizing currents sensitive to blockade by the $\alpha 7$ nAChR-selective antagonists α -bungarotoxin (α -BGT), methyllycaconitine (MLA), and α -conotoxin-ImI, and show an intracellular Mg^{2+} -dependent inward rectification and a rundown that is associated with the intracellular high-energy phosphate compounds. In contrast to type IA currents, type II and III currents, which desensitize very slowly, can be recorded from a small population of the hippocampal neurons and can be differentiated from one another on the basis of their sensitivity to nicotinic antagonists. Activation of type II currents is inhibited by DH β E (100 nM), and that of type III currents is inhibited by mecamylamine (1 μ M) [2].

Although nAChR subtype-selective antagonists have been very helpful in assigning a receptor subtype to a nicotinic response, the recent discovery that choline modulates the function and expression of neuronal nAChRs made up of the $\alpha 7$ subunit introduced a key pharmacological tool to distinguish $\alpha 7$ nAChRs from other nAChR subtypes [4, 8]. In cultured hippocampal neurons, choline fully activates $\alpha 7$ nAChR-mediated type IA currents with an apparent EC_{50} of 1.6 mM, does not evoke $\alpha 4\beta 2$ nAChR-mediated type II currents, and induces $\alpha 3\beta 4$ nAChR-mediated type III currents with 20% of the apparent efficacy of acetylcholine (ACh). Also, when continuously applied to these neurons, choline, like other nicotinic agonists, desensitizes the $\alpha 7$ nAChRs subserving type IA currents with an IC_{50} of 37 μ M [4]. Therefore, choline as a nicotinic agonist has the unique capability of providing substantial clues regarding the nAChR subtype subserving a given nicotinic response.

Using drug-delivery systems that allow for agonists to be rapidly applied to the vicinity of neurons and equally rapidly removed, and infrared-assisted videomicroscopy, which allows for visualization of individual neurons in slices, initial studies from our laboratory showed that neuronal nAChRs located in preter-

minal sites of CA1 interneurons facilitate γ -amino butyric acid (GABA) release [3]. Answering the question regarding the nAChR subtype involved in modulating the release of GABA from CA1 interneurons became of utmost importance given that synchronization of the hippocampal neuronal activity has been shown to be enforced by the ongoing activity in the interneurons [6]. Thus, using a number of nAChR-subtype-selective pharmacological tools, particularly choline, we demonstrated that in hippocampal slices: i) CA1 interneurons can express functional $\alpha 7$ and $\alpha 4\beta 2$ nAChRs; ii) $\alpha 7$ nAChRs are located predominantly in the somato-dendritic region of the CA1 interneurons, whereas $\alpha 4\beta 2$ nAChRs are presently mostly in preterminal regions of these interneurons; and iii) both $\alpha 7$ and $\alpha 4\beta 2$ nAChRs can facilitate the release of GABA from the interneurons [5]. Such findings provide direct support to previous studies in which a GABAergic mechanism was suspected to explain the relationship between the $\alpha 7$ nAChR gene locus and attentional deficits in schizophrenia [7], and in which cholinergic deafferentation was linked to kindling epileptogenesis [10]. In addition, these results indicate that choline, by controlling the function and expression of $\alpha 7$ nAChRs in the CNS may have a critical role in modulating the activity of the CA1 interneurons, and, ultimately, the excitability of the CA1 pyramidal neurons.

The finding that choline (a metabolite of the ACh hydrolysis) can control the function and expression of neuronal nAChRs bearing $\alpha 7$ subunits and that these receptors are capable of modulating differently the release of a variety of neurotransmitters raised the question of whether anticholinesterases, including the organophosphate (OP) nerve agents VX, soman, and sarin, can alter transmitter release from CNS neurons simply by altering the levels of choline and ACh. By means of the patch-clamp technique, we have recently demonstrated that at toxicologically relevant concentrations (≥ 0.1 nM), VX and sarin, but not soman, increase both tetrodotoxin (TTX)-sensitive and -insensitive release of glutamate and GABA from hippocampal neurons. The effects of VX and sarin are unrelated to cholinesterase inhibition and to alterations of the function of presynaptic nAChRs. In fact, the effect of VX on TTX-sensitive transmitter release appears to be mediated by its direct interaction with voltage-gated Na^+ channels. These studies altogether demonstrate the physiological relevance of nAChRs and choline in controlling synaptic function in the CNS and may provide the basis for a better understanding of the actions underlying the neurotoxic effects of many OPs, including the nerve agent VX.

References

- 1 Alkondon M. and Albuquerque E.X., Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. I. Pharmacological and functional evidence for distinct structural subtypes. *J Pharmacol Exp Ther* 265 (1993) 1455–1473.
- 2 Alkondon M., Reinhardt S., Lobron C., Hermesen B., Maelicke A. and Albuquerque E.X., Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. II. The rundown and inward rectification of agonist-elicited whole-cell currents and identification of receptor subunits by *in situ* hybridization. *J Pharmacol Exp Ther* 271 (1994) 494–506.

- 3 Alkondon M., Pereira E.F.R., Cortes W.S., Maelicke A. and Albuquerque E.X., Choline is a selective agonist of $\alpha 7$ nicotinic acetylcholine receptors in rat brain neurons. *Eur J Neurosci* 9 (1997) 2734–2742.
- 4 Alkondon M., Pereira E.F.R., Barbosa C.T.F. and Albuquerque E.X., Neuronal nicotinic acetylcholine receptor activation modulates γ -aminobutyric acid release from CA1 neurons of rat hippocampal slices. *J Pharmacol Exp Ther* 283 (1997) 1396–1411.
- 5 Alkondon M., Pereira E.F.R., and Albuquerque E.X., Choline and selective antagonists identify multiple subtypes of nicotinic acetylcholine receptors that modulate GABA release from CA1 interneurons in rat hippocampal slices. Submitted to *J. Neurosci.*, 1998.
- 6 Cobb S.R., Buhl E.H., Halasy K., Paulsen O. and Somogyi P., Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons. *Nature* 378 (1995) 75–78.
- 7 Freedman R., Coon H., Myles-Worsley M., Orr-Urtreger A., Olincy A., Davis A., Polymeropoulos M., Holik J., Hopkins J., Hoff M., Rosenthal J., Waldo M.C., Reimherr F., Wender P., Yaw J., Young D.A., Breese C.R., Adams C., Patterson D., Adler L.E., Kruglyak L., Leonard S., and Byerler W., Linkage of neurophysiological deficit in schizophrenia to a chromosome 15 locus. *Proc Natl Acad Sci USA* 94 (1997) 587–592.
- 8 Papke R.L., Bencheriff M. and Lippello P., An evaluation of neuronal nicotinic acetylcholine receptor activation by quaternary nitrogen compounds indicates that choline is selective for the $\alpha 7$ subtype. *Neurosci Lett* 213 (1996) 201–204.
- 9 Petit T.L., The neurobiology of learning and memory: Elucidation of the mechanism of cognitive function. *NeuroToxicology* 5 (1988) 413–428.
- 10 Tóth K., Freund T.F. and Miles R., Disinhibition of rat hippocampal pyramidal cells by GABAergic afferents from the septum. *J Physiol (Lond)* 500 (1997) 463–474.
- 11 Schröder H., Giacobini E., Struble R.G., Zilles K., and Maelicke A., Nicotinic cholinergic neurons of the frontal cortex are reduced in Alzheimer's disease. *Neurobiol. Aging* 12 (1991) 259–262.

2

Sensitivity of bovine retinal acetylcholinesterase to phenserine: A derivative of physostigmine

A.A. Al-Jafari^a, M.A. Kamal^b, N.H. Greig^b

^aDepartment of Biochemistry, College of Science, King Saud University, Riyadh, Saudi Arabia

^bMolecular Physiology and Genetics Section, National Institute of Aging, National Institute of Health, Baltimore, Maryland, USA

Currently, there has been a keen interest in the cholinesterases inhibitors, because of their therapeutic importance in the treatment

of dementia disorders, such as Alzheimer's and other cholinergic-related impairments (Messamore et al., 1993; Smith and Swash, 1979; Becker and Giacobini, 1988). These inhibitors enhance the cholinergic response by inhibition of acetylcholinesterase (AChE). Phenserine, a derivative of the AChE inhibitor physostigmine, acts as a long lasting inhibitor of the central nervous system and may be used for the treatment of Alzheimer's disease in the future (Greig et al., 1995).

Recently, we have evaluated the inhibition of human erythrocyte AChE activity by phenserine (Al-Jafari et al., 1998). In the present study, we have determined the sensitivity of bovine retinal AChE to this drug and compared it with human erythrocyte AChE inhibition. The phenserine has inhibited bovine retinal AChE activity in a concentration-dependent manner. The IC_{50} for retinal AChE was found to be 0.249 μM (figure 1) and for human erythrocyte it has been reported to be 0.045 μM . These values indicate that the retinal AChE is 5.53 times less sensitive than human erythrocyte AChE.

References

- Messamore E, Warpman U, Ogane N, Giacobini E (1993) Cholinesterase inhibitor effects on extracellular acetylcholine in rat cortex. *Neuropharmacol* 32, 745–750
- Smith CM, Swash M (1979) Physostigmine in Alzheimer's disease. *Lancet* 1, 42–46
- Becker R, Giacobini E (1988) Mechanisms of cholinesterase inhibition in senile dementia of the Alzheimer type: clinical, pharmacological, and therapeutic aspects. *Drug Dev Res* 12, 163–195
- Greig N, Pei X-F, Soncrant TT, Ingram DK, Brossi A (1995) Phenserine and ring C hetero-analogues: Drug candidates for the treatment of Alzheimer's disease. *Med Res Rev* 15, 3–31
- Al-Jafari AA, Kamal MA, Greig NH, Alhomida AS, Perry ER (1998) Kinetics for the inhibition of human erythrocyte acetylcholinesterase by a novel derivative of physostigmine: Phenserine (submitted)

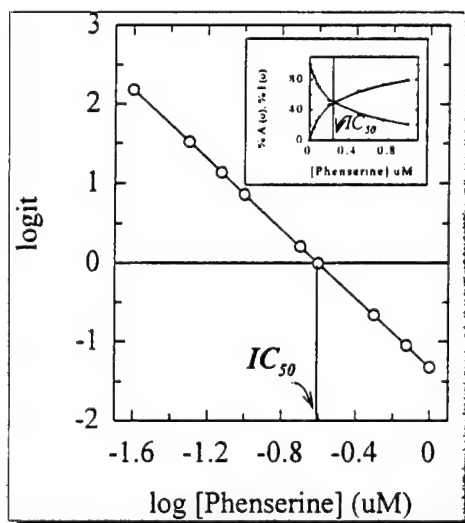


Figure 1. Inhibition of bovine retinal AChE by phenserine. Transformed data are presented in the form of a plot, where Logit = \ln [% activity / % inhibition]. Correlation coefficient is 0.9988. Each point represents the mean of triplicate experiments. Inset represents a novel way of determination of IC_{50} by combination of two conventional methods in same plot, i.e., plot of percentage activity of AChE and percentage inhibition of AChE versus log phenserine concentrations.

3

Determinants of channel gating located in the N-terminal extracellular domain of nicotinic $\alpha 7$ receptor

R. Anand^a, V. Gerzanich^b, M.E. Nelson^b, G.B. Wells^b, J. Lindstrom^b

^aNeuroscience Center of Excellence & Department of Biochemistry and Molecular Biology, LSU Medical Center, New Orleans, LA 70112, USA

^bDepartment of Neuroscience, University of Pennsylvania Medical School, Philadelphia, PA 19104, USA

The $\alpha 7$, $\alpha 8$, and $\alpha 9$ subunits of the AChR family form functional homomers when expressed in *Xenopus* oocytes (Couturier et al., 1990; Elgoyhen et al., 1994; Gerzanich et al., 1994; Schoepfer et al., 1990). We have taken advantage of the structural homogeneity of the two closely related homomeric $\alpha 7$ and $\alpha 8$ AChRs, in conjunction with their pharmacological differences, to identify regions that affect activation of these AChRs.

By single channel analysis of $\alpha 7$ AChR currents, we show that the difference in efficacy between the two agonists acetylcholine (ACh) and 1,1-dimethyl-4-phenylpiperazinium (DMPP) is due to a slower channel activation rate by DMPP. Using chimeras of the two closely related subunits $\alpha 7$ and $\alpha 8$, we map residues that affect channel activation rate and agonist affinity to two different regions of the extracellular domain. Residues that affect channel activation rate are within the sequence 1179, whereas residues that affect agonist affinity are within the sequence 180–208. Collectively, our results demonstrate that regions within the N-terminal extracellular domain of $\alpha 7$ and $\alpha 8$ AChRs (and possibly other ligand-gated channels) govern not only the affinity

for agonists but also the transition rates between closed and open states.

References

- Couturier, S., Bertrand, D., Matter, J. M., Hernandez, M. C., Bertrand, S., Millar, N., Valera, S., Barkas, T., and Ballivet, M. (1990) A neuronal nicotinic acetylcholine receptor subunit ($\alpha 7$) is developmentally regulated and forms a homo-oligomeric channel blocked by α -BTX. *Neuron* 5: 847–856.
- Elgoyhen, A. B., Johnson, D. S., Boulter, J., Vetter, D. E., and Heinemann, S. (1994) $\alpha 9$: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell* 79: 705–715.
- Gerzanich, V., Anand, R., and Lindstrom, J. (1994) Homomers of $\alpha 8$ and $\alpha 7$ subunits of nicotinic receptors exhibit similar channel but contrasting binding site properties. *Mol Pharmacol* 45: 212–220.
- Schoepfer, R., Conroy, W. G., Whiting, P., Gore, M., and Lindstrom, J. (1990) Brain α -bungarotoxin binding protein cDNAs and MAbS reveal subtypes of this branch of the ligand-gated ion channel gene superfamily. *Neuron* 5: 35–48.

4

Rhythmically bursting activity in rat medial septum after selective lesion of the cholinergic medial septal neurons and during aging

E. Aparts^{a,b}, F.R. Poindessous-Jazat^b, M.H. Bassant^b

^aService de Physiologie, Hôpital Saint Antoine, 75012 Paris, France

^bLaboratoire de Physiopharmacologie du système Nerveux, INSERM U161, 2, rue d'Alésia, 75014 Paris, France

The medial septum and the diagonal band of Broca nuclei (MS-DBB) contain cholinergic and GABAergic neurons which project to the hippocampus. A significant proportion of these septohippocampal neurons (SHN) display a rhythmically bursting (RB) activity that 'paces' the hippocampal theta rhythm. RB activity in the septohippocampal pathway has attracted interest because there is evidence that it is involved in learning and memory. The neurochemical nature of septal RB neurons is not firmly established. Two populations of RB septal neurons have been identified: cells which lose RB activity after atropine might be cholinergic whereas cells which retain RB activity might be GABAergic (Stewart and Fox, 1989). One way to identify RB SHNs as cholinergic or GABAergic is to examine the bursting activity after selective lesion of one sub-population of the septal neurons. This was achieved in the first part of the this study by lesioning the cholinergic SHNs with the immunotoxin 192 IgG-saporin (Book et al., 1992).

Anatomical studies show that MS-DBB neurons are affected by aging (Armstrong et al., 1993). The number of cholinergic neurons and the immunoreactivity for the calcium binding protein parvalbumin (PARV) are reduced in aged rats (Krzykowski et al., 1995). In the second part of the study, septal activity was recorded in 3-, 23- and 30-month-old unanesthetized rats to look

for age-related alterations of RB activity. The question of the vulnerability of RB SHNs (cholinergic?) to aging was addressed by comparing 192 IgG-saporin lesioned and aged rats.

Methods

192 IgG-saporin-lesioned rats

Three-month-old Sprague-Dawley rats received bilateral injections of either 192 IgG-saporin or saline (control rats) in the lateral ventricle. Ten to 16 days after injection, experiments were performed in urethane anesthetized and unanesthetized rats. In the first case, rats were anesthetized and placed in a stereotaxic apparatus. The skull was opened over the MS-DBB. Recording electrodes for electroencephalogram (EEG) were placed in the dorsal hippocampus. SHN neurons were identified by their antidromic response to the fimbria-fornix stimulation. MS-DBB unitary activity was recorded from micropipettes filled with 1 M NaCl and 2% Pontamine Blue. Recording sites were marked by a local release of dye.

In the second case, MS-DBB neurons were recorded in the absence of anesthesia. A painless head-restraint system was implanted prior to the recording sessions. Electrodes were implanted in the hippocampus and in the neck muscles for recording elec-

tromyogram (EMG) Over a course of 7 days, the rat was gradually habituated to the restraint system. The recording procedure was similar to that described for urethane anesthetized rats. The state of arousal was monitored by EEG and EMG during the recording sessions. The neurons were subsequently classified in several groups according to the states of arousal (W, wakefulness; SWS, slow wave sleep; REM sleep, rapid eye movement sleep). Brain sections were stained with a 192-IgG monoclonal antibody for the detection of the low-affinity NGF receptors or with a PARV monoclonal antibody to identify the GABAergic neurons. A staining for AChE positive fibers in the region of the hippocampus was performed to check for the efficiency of the cholinergic lesion.

Aged rats

MS-DBB neurons were recorded in unanesthetized rats of 3-, 23- and 30-months of age using the method described above. In lesioned and aged rats, the analysis of the unitary activity included: 1) mean spontaneous activity; 2) temporal structure of the discharge (RB or non-RB) and frequency of the periodic events; 3) percentage of RB neurons; and 4) burst parameters.

Results

192 IgG-saporin-lesioned rats

Acetylcholinesterase (AChE) histochemistry revealed a near-complete loss of cholinergic septal neurons and of cholinergic fibers in the hippocampus. In urethane-anesthetized lesioned rats, the percentage of RB neurons decreased significantly as compared to controls (17% vs. 41% for SHNs, $P < 0.001$ and 5% vs. 19% for unidentified septal neurons, $P < 0.0001$). The axonal conduction velocity and the burst frequency of the SHNs which retained a RB activity were higher in lesioned as compared to control rats ($P < 0.001$ and 0.05). The number of spikes per burst was lower and the burst duration was shorter in lesioned rats ($P < 0.01$). The frequency and the amplitude of the urethane-resistant hippocampal theta were altered. In unanesthetized lesioned rats, no RB septal neurons were found during W, as compared to 25% in controls. Their number was also markedly reduced during REM sleep (9.7% vs. 38.5%, $P < 0.01$). Histochemistry in 192 IgG-saporin treated rats showed that neurons that still display RB activity were found in areas devoid of AChE-positive neurons but containing PARV-positive (presumably GABAergic) neurons (Apartis et al., 1998).

Aged rats

During W, the percentage of RB neurons was significantly lower in 23- and 30-month-old rats than in 3-month-old rats (25.6% and 27% vs. 43.8%, $P < 0.01$). This result is in agreement with data previously obtained in urethane anesthetized rats (Lamour et al., 1989). The frequency of RB activity decreased at the age of 23 months (5.8 vs. 6 Hz, $P < 0.01$), this change being more pronounced at 30 months (5.3 Hz, $P < 0.0001$). The burst pattern was mildly affected by age: small increases in the number of spikes per burst and in interspike intervals (n.s.) caused a lengthening of the burst duration. During REM sleep, the decrease of

RB activity occurred at a more advanced age than during W: the percentage of RB neurons and the frequency of the burst were unchanged at 23 months and decreased only at 30 months (47.6% vs. 80.5%, $P < 0.05$ and 6 vs. 6.8 Hz, $P < 0.01$). No RB activity was recorded during SWS irrespective of age. During W, the frequency of hippocampal theta decreased significantly in both 23- and 30-month-old rats ($P < 0.0001$). During REM sleep, the frequency remained unchanged in 23- but decreased in 30-month-old rats (6.3 vs. 7.0 Hz at 3 months, $P < 0.0001$). The age-related impairments of hippocampal theta and frequency of RB septal neurons followed the same tendency: they appeared earlier during W (23 months) as compared to REM sleep (30 months).

Conclusion

Our data show that RB activity is considerably reduced after selective lesion of the cholinergic MS-DBB neurons. They suggest that the large majority of the RB septal neurons are cholinergic and that the few neurons retaining RB activity in lesioned rats are GABAergic. Intracellular and whole cell patch-clamp recordings show that MS-DBB neurons display features that probably favour RB activity, among them are low-threshold Ca^{2+} conductances (Alvarez De Toledo and Lopez-Barnero, 1988) and inhibitory and excitatory currents with fast kinetics (Schneeggenburger et al., 1992). If the majority of RB septal neurons are cholinergic then the decrease of RB activity in aged rats might be the consequence of the death of cholinergic neurons. Surviving neurons show morphological signs of progressive degeneration. It is conceivable that functional properties are impaired in damaged neurons which are no longer able to maintain a normal RB activity.

References

- Alvarez De Toledo G, Lopez-Barnero J (1988) Ionic basis of the differential neuronal activity of guinea-pig septal nucleus studied *in vitro*. *J Physiol* 396, 399-415
- Apartis E, Poindessous-Jazat F, Bassant MH (1998) Loss of rhythmically bursting neurons in the rat medial septum following selective lesion of the septohippocampal cholinergic system. *J Neurophysiol* 79, 1633-1642
- Armstrong DM, Sheffield R, Buzsáki G, Chen KS, Hersh LB, Nearing B, Gage FH (1993) Morphologic alterations of choline acetyltransferase-positive neurons in the basal forebrain of aged behaviorally characterized Fisher 344 rats. *Neurobiol Aging* 14, 457-470
- Book AA, Wiley RG, Schweitzer JB (1992) Specificity of 192 IgG-saporin for NGF receptor-positive cholinergic basal forebrain neurons in the rat. *Brain Res* 590, 350-355
- Krzywkowski P, de Bilbao F, Senut MC, Lamour Y (1995) Age-related changes in parvalbumin- and GABA-immunoreactive cells in the rat septum. *Neurobiol aging* 16, 29-40
- Lamour Y, Bassant MH, Jobert A, Joly M (1989) Septo-hippocampal neurons in the aged rat: Relation between their electrophysiological and pharmacological properties and behavioural performances. *Neurobiol Aging* 10, 181-186
- Schneeggenburger R, Lopez-Barnero J, Konnerth A (1992) Excitatory and inhibitory synaptic currents and receptors in rat medial septal neurons. *J Physiol* 445, 261-276
- Stewart M, Fox SE (1989) Two populations of rhythmically bursting neurons in the rat medial septum are revealed by atropine. *J Neurophysiol* 61, 982-993

5

An antibody specific for the neuronal nicotinic acetylcholine receptor alpha 4 subunit: High levels of expression in monoaminergic neurons

M.M. Arroyo-Jimenez^{a,b}, J.P. Bourgeois^a, A.M. Le Sourd^a, A. Fairen^b, J.P. Changeux^a

^aNeurobiologie Moléculaire, Biotechnologie, Institut Pasteur, 75724 Paris cedex 15, France

^bInstituto Cajal, CSIC, Madrid, Instituto de Neurociencias, Univ. Miguel Hernandez, Spain

In the brain, neuronal acetylcholine receptors (nAChRs) comprise two major types of subunits, α and β subunits. Current understanding of nAChR expression and distribution in the brain relies on localization of individual subunit transcripts or in the localization of protein based on immunohistochemistry. Different lines of evidence indicate that, in the central nervous system, nAChRs composed of $\alpha 4$ and $\beta 2$ subunits are a majority among native nicotinic receptors.

We have attempted the immunohistochemical detection of the $\alpha 4$ subunit using a subunit-specific antibody. We have used a polyclonal antibody raised against a peptide sequence corresponding to the cytoplasmic loop region of the rat $\alpha 4$ neuronal nicotinic acetylcholine receptor, comprising residues 573–592 of the cDNA deduced sequence. First, we assessed the specificity of this antibody using different approaches. Western blot analysis of rat brain homogenates and membrane extracts of cells transfected with $\alpha 4$ and $\beta 4$ cDNAs of nAChR subunits revealed a band of labelled protein with a molecular mass of 70 kDa.

Subsequently, we used this antibody to study the distribution of this subunit in the transfected cells and the rat brain tissue by light- and electron microscope immunohistochemistry. The immunoreaction product was mainly located in the cytoplasm and den-

drites, with varying intensity. The distribution of $\alpha 4$ like-immunoreactive ($\alpha 4$ -IR) cell bodies correlated well with previous in situ hybridisation results. Particularly strong labelling was detected in the monoaminergic cell groups in the brainstem. These included the dopaminergic cell areas such as the substantia nigra pars compacta and ventral tegmental area, the serotonergic neurons of the raphe nuclei and the noradrenergic neurons of the locus coeruleus. Transmitter identity of the $\alpha 4$ -positive neurons was tested by double-labelling sections of the rat midbrain both for $\alpha 4$ and for tyrosine hydroxylase, the rate-limiting enzyme responsible for the synthesis of dopamine, or for $\alpha 4$ and serotonin.

Using the DAB technique at the electron microscope level, $\alpha 4$ -IR structures comprised the cytoplasm and, particularly, the rough endoplasmic reticulum. In some dendrites, the immunoreactivity was found for the first time in association with postsynaptic densities. Since we observed in co-localisation experiments that $\alpha 4$ coexists with tyrosine hydroxylase in the dopaminergic cells of the substantia nigra, we suggest that nAChRs containing the $\alpha 4$ subunit may play a functional role at the postsynaptic level of nAChRs in these cells.

6

Acetylcholine synthesis activation and neuronal differentiation

G. Augusti-Tocco, F. Bignami, A. De Jaco, S. Biagioni

Dip. Biologia Cellulare e dello Sviluppo, Univ. 'La Sapienza', piazzale A. Moro, 5, 00185 Rome, Italy

Synaptogenesis has been a central problem in neurobiology for a long time; however, its mechanism is far from being clarified. Neuronal populations, in fact, utilise several transmitter molecules and receptors and follow different time-scales for the acquisition of their specific functions. Production of acetylcholine has been reported to occur in the early stages of the motor neuron development (Berg, 1978; Vaca, 1988); on the other hand a possible role of neurotransmitters in directing the early events of neuron/target interaction has also been postulated (Lipton and Kater, 1989).

Mouse neuroblastoma N18TG2 clone and the derived neuroblastoma \times glioma hybrid 108CC15 line provide a useful experimental model to analyse the role of the activation of neurotransmitter synthesis in the progression along neuronal differentiation pathways. In fact, the inability of the former clone to establish synaptic contacts is overcome in the hybrid line and the accomplishment of this more advanced differentiation phenotype is accompanied by the induction of choline acetyltransferase (ChAT) expression, which is actively synthesised in the hybrid, while it is nearly undetectable in the parental line (Hamprecht, 1977). With the aim to establish whether induction of ChAT expression modifies the expression of other specific neuronal markers we have transfected N18TG2 cells with a cDNA construct

for ChAT gene (Ishii et al., 1990) and characterised the isolated clones, with high ChAT activity, for their ability to express other neurospecific features (Bignami et al., 1997).

The isolated clones express different ChAT levels, as demonstrated by biochemical assay, Northern blot analysis and immunocytochemical staining. The ability of these clones to synthesize acetylcholine was also demonstrated by HPLC on cellular extract. Northern blot analysis shows increased expression in the transfected clones of mRNAs for neuronal proteins, associated to synaptic vesicle, such as synapsin I. Fiber outgrowth of transfected clones has also been evaluated to establish whether there is any relation between ChAT levels and morphological differentiation. This analysis shows that the transfected clone 1/2, not expressing ChAT activity, displays a very immature morphology and its ability to extend fibers remains rather poor also in the presence of 'differentiation' agents, as retinoic acid. On the other hand clones 2/4, 3/1 and 3/2, exhibiting high ChAT levels, display higher fiber outgrowth as compared to clone 1/2 both in the absence or presence of differentiating agents (figure 1).

Over the last few years a number of data have become available suggesting that, even at early developmental stages, neurotransmitter molecules can be spontaneously released and may act as regulators

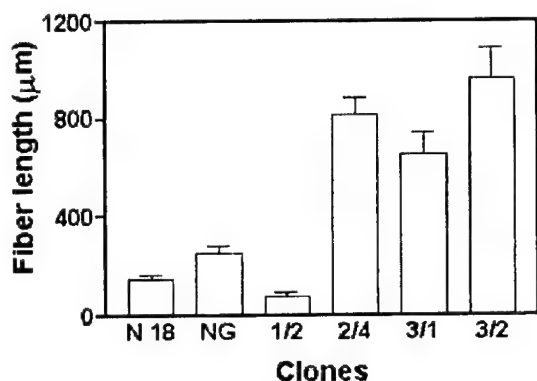


Figure 1. Quantification of process length per microscopic field in cultures maintained in the presence of 10^{-6} M retinoic acid for 4 days. N18, N18TG2 cells; NG, 108CC15 hybrid cells; 1/2, transfected clone not expressing ChAT activity; 2/4, 3/1 and 3/2, transfected clones expressing ChAT activity.

Table I. Secreted AChE activity in the culture medium.

Clones	Secreted AChE
aCS5	114.46 ± 9.83
CS37	31.5 ± 0.86
CS42	44.67 ± 1.46
CS43	63.15 ± 4.66

Comparison of AChE secretion in cultures of different clones. AChE release was measured over a 24-h period and was expressed as percent of the cellular content. Values are the mean ± SEM of at least 10 observations. aCS5: transfected clone not expressing ChAT activity; CS37, CS42 and CS43: transfected clones expressing ChAT activity.

of morphogenetic events (Lipton and Kater, 1989). AChE has also been reported as a regulator of neurite outgrowth, and various mechanisms for its action have been proposed (Small et al., 1995). Previous data showing changes of AChE cellular localization and secretion, related to developmental events (Biagioni et al., 1995; Coleman and Taylor, 1996), would be in favour of such hypothesis. It thus appeared of interest to perform a comparative study of AChE secretion in the different isolated clones (table I).

Comparison of AChE secretion in cultures of transfected clones showed that AChE release was significantly higher in aCS5

transfected clone not expressing ChAT activity as compared with the CS37, CS42 and CS43 transfected clones expressing ChAT activity. Sequential extraction of AChE activity revealed that the activity extracted in the detergent soluble fraction was considerably higher in the transfected clones expressing ChAT activity (50–55% of the total activity) than in the transfected clone not expressing ChAT activity (about 30%). The results obtained with the sequential extraction procedure support the hypothesis of a shift in the ChAT positive clones from a cytoplasmic to membrane association of the enzyme. Northern blot analysis of the different clones finally showed that the level of AChE mRNA was similar for all clones.

In conclusion these observations are in favour of a relation among activation of neurotransmitter synthesis and progression in the neuronal differentiation pathway. (This work was supported by funds from Fondazione Cenci-Bolognetti, MURST and CNR.)

References

- Berg DK (1978) Acetylcholine synthesis by chick spinal cord neurons in dissociated cell culture. *Dev Biol* 66, 500–512
- Biagioni S, Bevilacqua P, Scarsella G, Vignoli AL, Augusti-Tocco G (1995) Characterization of acetylcholinesterase secretion in neuronal culture and regulation by high K^+ and soluble factors from target cells. *J Neurochem* 64, 1528–1535
- Bignami F, Bevilacqua P, Biagioni S, De Jaco A, Casamenti F, Felsani A, Augusti-Tocco G (1997) Cellular acetylcholine content and neuronal differentiation. *J Neurochem* 69, 1374–1381
- Coleman BA, Taylor P (1996) Regulation of acetylcholinesterase expression during neuronal differentiation. *J Biol Chem* 271, 4410–4416
- Hamprecht B (1977) Structural, electrophysiological, biochemical and pharmacological properties of neuroblastoma-glioma cell hybrids in cell culture. *Int Rev Cytol* 49, 99–170
- Ishii K, Oda Y, Ichikawa T, Deguchi T (1990) Complementary DNAs for choline acetyltransferase from spinal cords of rat and mouse: nucleotide sequences, expression in mammalian cells, and *in situ* hybridization. *Mol Brain Res* 7, 151–159
- Small DH, Reed G, Whitefield B, Nurcombe V (1995) Cholinergic regulation of neurite outgrowth from isolated chick sympathetic neurons in culture. *J Neurosci* 15, 144–151
- Lipton SA and Kater SB (1989) Neurotransmitter regulation of neuronal outgrowth plasticity and survival. *Trends Neurosci* 12, 265–270
- Vaca K (1988) The development of cholinergic neurons *Brain Res Rev* 13, 216–268

7

Kinetic and structural studies on the interaction of the anti-Alzheimer drug, ENA-713, with *Torpedo californica* acetylcholinesterase

P. Bar-On^{a, b}, M. Harel^b, C.B. Millard^a, A. Enz^c, J.L. Sussman^b, I. Silman^a

Departments of ^aNeurobiology and ^bStructural Biology, Weizmann Institute of Science, Rehovot 76100, Israel
^cNovartis Pharma, Basel CH4002, Switzerland

The anti-Alzheimer drug, (+)S-N-ethyl-3-[(1-dimethylamino)-ethyl]-N-methyl-phenylcarbamate (ENA-713; ExelonTM) belongs to a series of miotone derivatives all displaying AChE inhibitory activity towards acetylcholinesterase (AChE) both in vitro and in vivo (Weinstock et al., 1986). Compared with other clinically use-

ful carbamates, ENA-713 has a longer duration of action in vivo, and preferentially inhibits AChE of the brain cortex and hippocampus (Enz et al., 1992, 1993). In order to clarify the basic mechanism of inhibition of AChE by ENA-713, we studied its reactions with *Torpedo californica* (Tc) AChE in vitro. The ap-

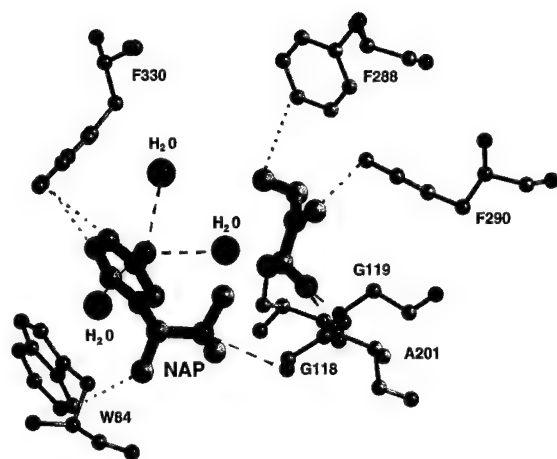


Figure 1. Close-up view of the active site of *TcAChE* after inhibition with ENA-713, with the individual amino acid residues labelled for orientation. The leaving group, NAP, is bound by interactions with the protein and with three water molecules.

parent bimolecular rate constant for progressive inhibition was low: $K_i = 6 \text{ M}^{-1}\text{min}^{-1}$ in 0.067 M Na/K phosphate buffer, pH 7.4, at 25 °C. The kinetics of reactivation were slow and complex, and displayed a substantial irreversible component. In contrast, *TcAChE* inhibited by ethylmethylcarbamylchloride, in which the bulky aromatic leaving group, 3-[(1-dimethylamino)ethyl]phenol (NAP), is replaced by chloride, reactivated much faster and quantitatively upon dilution. The apparent reversible binding constant

for *TcAChE* with ENA-713 was 200 μM , whereas NAP had a K_i of 0.5 μM , at pH 7.4 and 25 °C. Thus, the product bound much more tightly to *TcAChE* than did the intact carbamate in the reversible complex. Trigonal crystals of *TcAChE* were soaked with ENA-713, and the structure was solved and refined to 2.2 Å resolution. The refinement showed that Ser200 of *TcAChE* was ethylmethylcarbamylated, and NAP was bound non-covalently in the active site (figure 1). Significant contacts of NAP included hydrophobic interactions with Phe330 and Trp84, and its orientation was significantly different from that of edrophonium in the edrophonium-*TcAChE* complex (Harel et al., 1993). We conclude that ENA-713 can inhibit AChE by two mechanisms: 1) covalent carbamylation followed by slow, partial decarbamylation; and 2) action of the carbamate as a pro-drug to deliver the leaving group, NAP, which is itself a good reversible inhibitor of AChE.

References

- Enz A, Chappuis A, Probst A (1992) Different influence of inhibitors on acetylcholinesterase molecular forms G1 and G4 isolated from Alzheimer's disease and control brains. In: *Multidisciplinary Approaches to Cholinesterase Functions* (Shafferman A, Velan B, eds), pp 243–249, Plenum Press, New York
- Enz A, Amstutz R, Boddeke H, Gmelin G, Malanowski J (1993) Brain-selective inhibition of acetylcholinesterase: a novel approach to therapy for Alzheimer's disease. In: *Progress in Brain Research*, Vol. 98 (Cuello AC, ed), pp 431–438, Elsevier, Amsterdam
- Harel M, Schalk I, Ehret-Sabatier L, Bouet F, Goeldner M, Hirth C, Axelsen P, Silman I, Sussman JL (1993). Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase. *Proc Natl Acad Sci USA* 90, 9031–9035
- Weinstock M, Razin M, Chorev M, Tashma Z (1986) Pharmacological activity of novel anticholinesterase agents of potential use in the treatment of Alzheimer's disease. In: *Advances in Behavioral Biology*, Vol. 29 (Fisher A, Hanin I, Lachman P, eds), pp 539–549, Plenum Press, New York

8

Stimulation of muscarinic receptors in vivo induces dramatic modifications of the subcellular distribution of the m2 muscarinic receptor in striatal interneurons

V. Bernard^a, O. Laribi^a, A.I. Levey^b, B. Bloch^a

^aCentre National de la Recherche Scientifique, Unité Mixte de Recherche 5541, Laboratoire d'Histologie-Embryologie, Université Victor Segalen-Bordeaux 2, 146, rue Léo-Saignat, 33076 Bordeaux cedex, France

^bEmory University WMRB, Suite 6113, 1639 Pierce Dr., Atlanta, GA 30322, USA

Experiments on cells transfected in vitro with G-protein coupled receptors demonstrated that their stimulation by agonists induces different events including phosphorylation, endocytosis of the receptor, dissociation of the ligand from the receptor, dephosphorylation and either degradation of the receptor or recycling to the plasma membrane (Koenig and Edwardson, 1997). However, the mechanisms regulating, in vivo, the compartmentalization and recycling of receptors in neurons and their control by neurotransmitters in physiological, experimental and pathological circumstances are still poorly understood (Dumartin et al., 1998).

The aim of our study was to determine how the cholinergic environment influences the subcellular localization of m2R in striatal interneurons in vivo by using immunohistochemical approaches at light and electron microscopic level (Levey et al., 1995; Bernard et al., 1997). 1) We have examined the cellular and subcellular distribution of m2R in striatal neurons of control animals. 2) We have

studied the effect of the stimulation of muscarinic receptors with agonists on the localization of m2R at cellular and subcellular levels and we have determined the time course of this effect. In order to better understand the fate of the receptor after its own activation, we have quantified by using image analysis at electron microscopic level, the modifications of the distribution of m2R gold immunolabelling in the different subcellular organelles. 3) We have determined in these conditions if there is a specificity of internalization of a receptor by its own ligand or if a ligand is able to induce endocytosis of heteroreceptors. For this purpose, we have compared the distribution of the substance P receptor (SPR) and m2R after stimulation of muscarinic receptors.

We have shown that:

1) In control animals, m2R is expressed in cholinergic and NPY/somatostatin interneurons as auto- and heteroreceptors, respectively. In these neurons, m2R is located mostly at the plasma

membrane in perikarya and in dendrites. Presynaptic receptors have been detected in boutons. These data suggest that m2Rs are well-localized to modulate especially the activity of the cholinergic neuron including the electric activity and/or the release of Ach (Billard et al., 1995; Rouse et al., 1997). The m2Rs were usually detected at extrasynaptic sites, albeit they could be found rarely in association with symmetrical synapses, suggesting that the cholinergic transmission mediated by m2R occurs through synaptic and non-synaptic mechanisms.

2) The stimulation of muscarinic receptors with oxotremorine provokes a decrease of the density of m2R at the membrane (-63%) and an increase of those associated with endosomes (+86%) in perikarya. This suggests that the stimulation of m2R induces endocytosis of m2R. Internalization of m2R into endosomes has been shown in dendrites as well. The treatment induces a very strong increase of m2R immunoparticles associated with multivesicular bodies in perikarya and dendrites and thus could be the sign of an increase of the degradation of m2R. We have also shown a slight increase of m2R in the Golgi apparatus (+26%), but not in the endoplasmic reticulum, suggesting that the stimulation of muscarinic receptors may have an effect on the way of synthesis of m2R. We have demonstrated that internalization starts as soon as 3 min after injection and lasts for more than 3 h. Since our quantification has been performed 45 min after injection of oxotremorine, we cannot exclude that other phenomena (neosynthesis, etc.) occur with a different time course. In contrast, the stimulation of muscarinic receptors with pilocarpine has no effect on the distribution of m2R, probably due to the lower specificity for m2R of this drug compared to oxotremorine.

3) We have shown by double immunofluorescence that SPR, a G-protein coupled receptor, is co-localized with m2R in cholinergic and somatostatin/NPY striatal interneurons. In control rats,

SPR and m2R are colocalized at the plasma membrane. In contrast, after oxotremorine, SPR stays at the membrane whereas m2R is internalized. This suggests that there is a specificity of internalization of a receptor by its own agonist.

Our data demonstrate for the first time that the cholinergic environment influences in vivo the subcellular distribution, the addressing and the receptor availability of m2R in striatal neurons as auto- and heteroreceptor. The different events following the stimulation of the receptors, including endocytosis, degradation and/or neosynthesis may have a key role in the function of striatal interneurons, especially in neurotransmitter release, and/or electric activity. Our data demonstrate also that the cholinergic transmission may occur through synaptic as well as non-synaptic mechanisms.

References

- Bernard V, Somogyi P, Bolam JP (1997) Cellular, subcellular and subsynaptic distribution of AMPA-type glutamate receptor subunits in the neostriatum of the rat. *J Neurosci* 17, 819-833
- Billard W, Binch Hr, Crosby G, McQuade RD (1995) Identification of the primary muscarinic autoreceptor subtype in rat striatum as m2 through a correlation of in vivo microdialysis and in vitro receptor binding data. *J Pharmacol Exp Ther* 273, 273-9
- Dumartin B, Caille I, Gonon F, Bloch B (1998) Internalization of D1 dopamine receptor in striatal neurons in vivo as evidence of activation by dopamine agonists. *J Neurosci* 18, 1650-61
- Koenig JA, Edwardson JM (1997) Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol Sci* 18, 276-87
- Levey AI, Edmunds SM, Hersch SM, Wiley RG, Heilman CJ (1995) Light and electron microscopic study of m2 muscarinic acetylcholine receptor in the basal forebrain of the rat. *J Comp Neurol* 351, 339-56
- Rouse ST, Thomas TM, Levey AI (1997) Muscarinic acetylcholine receptor subtype, m2: diverse functional implications of differential synaptic localization. *Life Sci* 60, 1031-8

9 Regulation of the expression of the gene encoding the rat vesicular acetylcholine transporter: Role of an upstream region

S. Berrard, L. Houhou, Y. Oda, S. De Gois, E. Thevenot, F. Pajak, R. Cervini, G. Vodjdani, J. Mallet

CNRS, Laboratoire de Génétique de la Neurotransmission et des Processus Neurodégénératifs, Hôpital Pitié-Salpêtrière, 75013 Paris, France

Choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VACHT) are encoded by two embedded genes, the intronless coding sequence of VACHT lying within the first intron of the ChAT gene. The structure of the ChAT and VACHT genes is conserved from worm to man and defines a so-called 'choli-

nergic gene locus' (Alfonso et al., 1994; Bejanin et al., 1994; Erickson et al., 1994; Naciff et al., 1997). In mammals, this organization is unusual for two genes whose products are both specifically required for the expression of the same neuronal phenotype. It suggests that transcription for both genes is coor-

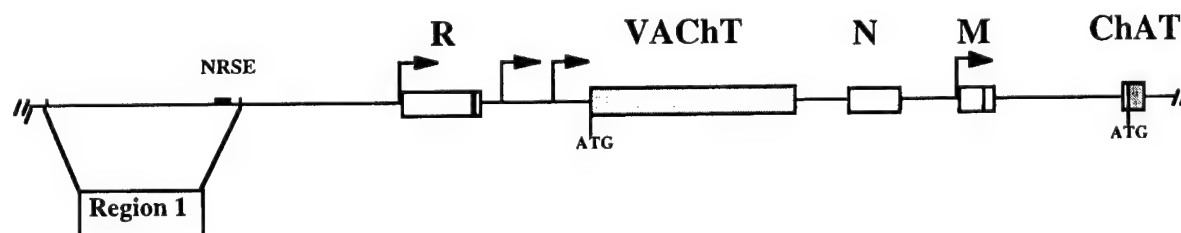


Figure 1. Schematic representation of the 5' region of the rat ChAT gene and of the VACHT coding sequence. Open and grey filled boxes indicate non-coding and coding sequences, respectively. The black box represents the neuron-restrictive silencer element (NRSE). R, N and M are the three ChAT non-coding exons (Kengaku et al., 1993). Arrows represent the promoter regions identified in the cholinergic gene locus.

dinately regulated.

To dissect the regulatory mechanisms of ChAT and VAcHT gene expression, the identification of the promoter regions of the cholinergic gene locus was required. In rodent, two promoters of the ChAT gene have been localized upstream from exons R and M respectively (figure 1; Ibanez and Persson, 1991; Bejanin et al., 1992; Misawa et al., 1992). We then identified rat VAcHT transcripts containing the exon R, suggesting that ChAT and VAcHT genes share a common upstream promoter (Bejanin et al., 1994). Moreover, we found two promoter regions of the VAcHT gene downstream from exon R, which are active in cholinergic as well as in non-neuronal cells (figure 1; Cervini et al., 1995).

In the present study, we have investigated the regulatory mechanisms that restrict the activity of the rat VAcHT promoters to cholinergic cells. We particularly focused on a genomic region of potential regulatory interest, designated as Region 1 and situated upstream from the R-type promoter (figure 1). This 2343-bp region has been shown to direct the expression in vivo of a heterologous promoter in cholinergic structures (Lönnerberg et al., 1995). Moreover, it contains a neuron-restrictive silencer element (NRSE) that interacts with a neuron-restrictive silencer factor to repress the activity of a downstream heterologous promoter in non-cholinergic cells (Lönnerberg et al., 1996). Transient transfection experiments of different types of cell lines reveal that Region 1 contributes to the cell specific regulation of the activity of the VAcHT promoters, but is not sufficient to specifically restrict their activity to cholinergic cells. Experiments are in progress to determine whether the NRSE itself is required for the regulation of the VAcHT gene expression.

References

- Alfonso A, Grundahl K, McManus JR., Asbury JM, Rand JB (1994) Alternative splicing leads to two cholinergic proteins in *Caenorhabditis elegans*. *J Mol Biol* 241, 627-630.
- Bejanin S, Habert E, Berrard S, Dumas Milne Edwards JB, Loeffler JB, Mallet J (1992) Promoter elements of the rat choline acetyltransferase gene allowing nerve growth factor inducibility in transfected primary cultured cells. *J Neurochem* 58, 1580-1583?
- Bejanin S, Cervini R, Mallet J, Berrard S (1994) A unique gene organization for two cholinergic markers, choline acetyltransferase and a putative vesicular transporter of acetylcholine. *J Biol Chem* 269, 21944-21947.
- Cervini R, Houhou L, Pradat PF, Bejanin S, Mallet J, Berrard S (1995) Specific vesicular acetylcholine transporter promoters lie within the first intron of the rat choline acetyltransferase gene. *J Biol Chem* 270, 24654-24657.
- Erickson JD, Varoqui H., Schäfer MK, Modi W, Diebler MF, Weihe E, Rand J., Eiden L E, Bonner TI, Usdin TB (1994) Functional identification of a vesicular acetylcholine transporter and its expression from a "cholinergic" gene locus. *J Biol. Chem* 269, 21929-21932.
- Ibanez CF, Persson H (1991) Localization of sequences determining cell type specificity and NGF responsiveness in the promoter region of the rat choline acetyltransferase gene. *Eur J Neurosci* 3, 1309-1315.
- Kengaku M, Misawa H, Deguchi T (1993) Multiple mRNA species of choline acetyltransferase from rat spinal cord. *Mol Brain Res* 18, 71-76.
- Lönnerberg P, Lendahl V, Funakoshi H, Richter L, Persson H, Ibanez CF (1995) Regulatory region in choline acetyltransferase gene directs developmental and tissue-specific expression in transgenic mice. *Proc Natl Acad Sci USA* 92, 4046-4050.
- Lönnerberg P, Schoenherr CJ, Anderson DJ, Ibanez CF (1996) Cell type-specific regulation of choline acetyltransferase gene expression. *J Biol Chem* 271, 33358-33365.
- Misawa H, Ishii K, Deguchi T (1992) Gene expression of mouse choline acetyltransferase. Alternative splicing and identification of a highly active promoter region. *J Biol Chem* 267, 20392-20399.
- Naciff JM, Misawa H, Dedman JR (1997) Molecular characterization of the mouse vesicular acetylcholine transporter gene. *Neuroreport* 8, 3467-3473.

10 Effect of ciliary neurotrophic factor on cholinergic gene expression: Modulation by nerve growth factor and glucocorticoids.

B. Berse^a, I. López-Coviella^b, J. Krzysztof Blusztajn^{a, b}

^aDepartment of Pathology and Laboratory Medicine and ^bDepartment of Psychiatry, Boston University School of Medicine, Boston, MA 02118, USA

The two proteins essential for cholinergic neurotransmission, choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VAcHT), are encoded by the cholinergic locus, which exhibits unique genomic organization; the VAcHT coding sequence is contained within the first intron of the ChAT gene. The two genes share some regulatory promoter sequences and non-coding exons. We and others had demonstrated that the expression of these genes is coordinately regulated (Berrard et al., 1995; Berse and Blusztajn, 1995; Misawa et al., 1995; Berse and Blusztajn, 1997; Hill and Robertson, 1997; Matsuura et al., 1997; Sun et al., 1997), however, there are examples of physiological situations where only one is expressed, and not the other (Holler et al., 1996; Fujii et al., 1998; Kitamoto et al., 1998). Many extracellular factors, including neurotrophins, retinoids, glucocorticoids, and cytokines, have been shown to affect acetylcholine (ACh) production through the modulation of ChAT/VAcHT expression. Here we demonstrate that the activation of cholinergic

gene expression by ciliary neurotrophic factor (CNTF) is modulated by nerve growth factor (NGF) and glucocorticoids.

As a model, we use a hybrid cell line (SN56), generated by fusing septal primary neurons obtained from postnatal day 21 mice with murine neuroblastoma N18TG2 cells. SN56 cells have many features of septal cholinergic neurons, however they do not express the TrkA high affinity NGF receptor. For experiments involving NGF, we restored TrkA signaling in SN56 cells by stable transfection with the plasmid pDM115 (a gift from Dr. Moses Chao), carrying rat *trkA* cDNA under the control of the murine sarcoma virus long terminal repeat promoter. The plasmid contains the neomycin resistance gene under the control of the SV40 promoter, for selection with geneticin (G418). The transfected clones were maintained in medium with G418, and screened for TrkA expression by Western blotting. Two clones expressing high levels of TrkA, and one TrkA-negative transfectant were selected for further experiments.

Cells were grown in DME medium supplemented with 10% fetal bovine serum in the presence or absence of test compounds for varying time periods. For ACh measurements, the cells were incubated at 37 °C in medium supplemented with 5 μ M choline and 15 μ M neostigmine, for 1 h prior to collection. The content of ACh was determined by HPLC with an enzymatic reactor containing acetylcholinesterase and choline oxidase and an electrochemical detector. Northern blotting with mouse cDNA probes for ChAT and VACHT was performed as described previously (Berse and Blusztajn, 1995).

We found that the expression of ChAT and VACHT mRNA and ACh content are coordinately up-regulated by CNTF, glucocorticoids and NGF in SN56 cells. In addition, we showed that there is a synergistic effect of CNTF (20 ng/mL) and dexamethasone (a synthetic glucocorticoid, 1 μ M) on ChAT and VACHT mRNA expression. CNTF treatment of SN56 cells caused rapid (within 5 min) translocation of the transcription factor Stat3 to the nucleus and activation of specific binding to the CNTF response element, as evidenced by electrophoretic mobility shift assay. Pre-treatment of the cells with dexamethasone for various periods of time did not affect Stat3 translocation evoked by CNTF. Several recent studies addressed the mechanisms of the synergy between glucocorticoids and the IL-6 family of cytokines (of which CNTF is a member). The proposed models include glucocorticoid-enhanced expression of IL-6 signaling components (Nesbitt and Fuller, 1992), as well as direct complex formation between Stat3 and the glucocorticoid receptor leading to transcription co-activation through both cytokine- and glucocorticoid-responsive elements (Zhang et al., 1997). Experiments are in progress to determine if a similar complex is formed upon co-stimulation of SN56 cells with CNTF and dexamethasone.

Stimulation of SN56 TrkA transfectants with NGF activated two signaling pathways; one inducing tyrosine phosphorylation of the adapter protein Shc and MAP kinases ERK1 p44 and ERK2 p42, and one modulating the cytosolic free calcium concentration. In the transfectants, NGF (100 ng/mL for 48 h) increased ChAT/VACHT mRNA expression and acetylcholine production up to two-fold. NGF treatment also induced better attachment to the substratum and neurite formation. These responses did not occur in the untransfected SN56 cells or in a TrkA-negative transfectant, which express only the low affinity p75 NGF receptor; therefore we conclude that these actions of NGF require TrkA signaling. Surprisingly, the combined treatment of SN56 cells expressing TrkA with CNTF and NGF revealed that NGF down-regulated the stimulatory effect of CNTF on the expression of the cholinergic locus. This is probably not due to interference with Stat3 signaling, since pre-treatment with NGF for various time periods between 15 min to 48 h did not inhibit rapid Stat3 translocation evoked by CNTF. The results suggest that NGF affects the induction of cholinergic gene expression by CNTF through mechanisms other than interference with the JAK/STAT pathway.

References

- Berrard, S.H Varoqui,R Cervini,M Israel,J Mallet,MF Diebler (1995). Coregulation of two embedded gene products, choline acetyltransferase and the vesicular acetylcholine transporter. *J Neurochem* 65, 939-42.
- Berse, B, JK Blusztajn (1995). Coordinated up-regulation of choline acetyltransferase and vesicular acetylcholine transporter gene expression by the retinoic acid receptor alpha, cAMP, and leukemia inhibitory factor/ciliary neurotrophic factor signaling pathways in a murine septal cell line. *J Biol Chem* 270, 22101-4.
- Berse, B, JK Blusztajn (1997). Modulation of cholinergic locus expression by glucocorticoids and retinoic acid is cell-type specific. *FEBS Lett* 410, 175-9.
- Fujii, T,S Yamada,Y Watanabe,H Misawa,S Tajima,K Fujimoto,T Kasahara,K Kawashima (1998). Induction of choline acetyltransferase mRNA in human mononuclear leukocytes stimulated by phytohemagglutinin, a T-cell activator. *J Neuroimmunol* 82, 101-7.
- Hill, DP, KA Robertson (1997). Characterization of the cholinergic neuronal differentiation of the human neuroblastoma cell line LA-N-5 after treatment with retinoic acid. *Brain Res Dev Brain Res* 102, 53-67.
- Holler, T,B Berse,JM Cermak,MF Diebler,JK Blusztajn (1996). Differences in the developmental expression of the vesicular acetylcholine transporter and choline acetyltransferase in the rat brain. *Neurosci Lett* 212, 107-10.
- Kitamoto, T,W Wang,PM Salvaterra (1998). Structure and organization of the *Drosophila* cholinergic locus. *J Biol Chem* 273, 2706-13.
- Matsuura, J,K Ajiki,T Ichikawa,H Misawa (1997). Changes of expression levels of choline acetyltransferase and vesicular acetylcholine transporter mRNAs after transection of the hypoglossal nerve in adult rats. *Neurosci Lett* 236, 95-8.
- Misawa, H,R Takahashi,T Deguchi (1995). Coordinate expression of vesicular acetylcholine transporter and choline acetyltransferase in sympathetic superior cervical neurones. *Neuroreport* 6, 965-8.
- Nesbitt, JE GM Fuller (1992). Differential regulation of interleukin-6 receptor and gp130 gene expression in rat hepatocytes. *Mol Biol Cell* 3, 103-12.
- Sun, X,X Tian,JB Suszkiw (1997). Reduction of vesicular acetylcholine transporter mRNA in the rat septum following lead exposure. *Neuroreport* 8, 891-4.
- Zhang, Z,S Jones,JS Hagoood,NL Fuentes,GM Fuller (1997). STAT3 acts as a co-activator of glucocorticoid receptor signaling. *J Biol Chem* 272, 30607-10.

11 Glutamate receptor-mediated synaptic responses in rat hippocampus after cholinergic deafferentation by the immunotoxin 192 IgG-saporin. Possible implications for the aging brain.

J.M. Billard, A. Jouvenceau, P. Dutar

Laboratoire de Physiopharmacologie du Systeme Nerveux, Paris, INSERM U161, 2, rue d'Alesia, 75014 Paris, France

Alterations in both cholinergic and glutamatergic systems have been suggested to contribute to the deficit in memory functions occurring during normal aging and related diseases (McEntee and Crook, 1993; Muir, 1997). Nevertheless, the relative role of each component remains an open issue. In fact, the effect of glutamate on acetylcholine (ACh)-mediated responses is well documented

(see Giovannini et al., 1997 for review). On the contrary, less is known about a cholinergic influence on responses induced by glutamate receptor activation (Markram and Segal, 1990). In the present study, we studied the effects of lesioning the cholinergic septohippocampal fibres with the immunotoxin 192 IgG-saporin (Wiley et al., 1991) on the synaptic responses mediated by glu-

tamate in area CA1 of rat hippocampus. Concomitantly, these responses were recorded in aged animals to better assess the consequences of the cholinergic deficit in these animals.

Experiments were conducted in 3–4-month-old and 25–33-month-old male Sprague-Dawley rats, as well as in young rats treated with an intraventricular injection of 192 IgG-saporin (4 µg in 7.5 µL of saline). One animal was daily studied using extracellular recordings in the *ex vivo* slice (400 µm) preparation. The composition of the bath medium was (mM): NaCl, 119; KCl, 3; MgSO₄, 1.5; CaCl₂, 3; NaHCO₃, 26.2; NaH₂PO₄, 1.0; glucose, 11. Extracellular recordings were obtained from the apical dendritic layer of the CA1 hippocampal area using micropipettes filled with 2 M NaCl and having resistances of 2–6 MΩ. Presynaptic fibre volleys (PFVs) and field EPSPs (fEPSPs) were evoked by electrical stimulation (100 µs duration) of CA1 afferent fibres (Schaffer collaterals and commissural fibres) located in the stratum radiatum.

Input-output (I/O) curves were first studied in control medium in young (10 slices in five animals), 192 IgG-saporin pretreated (11 slices in three animals) and aged rats (seven slices in five animals). Stimulation of the stratum radiatum induced a PFV followed by a fEPSP, both increasing with the stimulus intensity. The fEPSPs were totally suppressed by CNQX (10 µM) thus reflecting the activation of non-N-methyl-D-aspartate receptors (non-NMDAR). The comparison of the I/O curves revealed that the PFV were not significantly altered in young and 192 IgG-saporin pretreated animals [$F(1,20) = 1.55$, $P = 0.23$] as well as in young and aged rats [$F(1,15) = 1.754$, $P = 0.2$]. However, when the fEPSP slopes were plotted against the slope of the PFVs, we found that the non-NMDAR-mediated responses were not statistically affected in rats with the cholinergic denervation of the hippocampus [$F(1,20) = 5.87$, $P = 0.17$]. On the contrary, fEPSPs were significantly depressed in aged animals [$F(1,15) = 6.229$, $P = 0.02$].

In another sets of experiments, I/O curves were constructed from slices perfused for at least 40 min with a Mg²⁺-free medium and where CNQX was added 20 min before the recordings. In these conditions, a fEPSP was induced which differed from that recorded in control medium by its slow onset, its prolonged duration and its sensitivity to 2-APV (30 µM). Comparison of the NMDAR-mediated synaptic responses revealed that both the amplitude [$F(1,25) = 42.3$, $P < 0.0001$] and the duration [$F(1,25) = 5.87$, $P = 0.023$] of NMDAR-mediated fEPSPs were significantly increased in 192 IgG-saporin pretreated rats. In aged rats, we found that the duration of these responses was significantly enhanced [$F(1,28) = 5.08$, $P = 0.03$] whereas the amplitude was not statistically altered [$F(1,29) = 0.001$, $P = 0.97$].

The present study first did not reveal significant alterations of non-NMDAR-mediated synaptic responses of CA1 pyramidal cells after lesion of cholinergic septohippocampal fibres by the immunotoxin 192 IgG-saporin. These responses were significantly depressed in aged rats (Barnes et al., 1992; Papatheodoropoulos and Kostopoulos, 1996). These results therefore indicate that the cholinergic deficit occurring in the aged hippocampus (see Muir, 1997) do not significantly contribute to the impaired activation of these glutamate subtypes of receptors. Accordingly, this impairment was suggested to result mainly from a decreased number of synaptic contacts between glutamatergic afferents and pyramidal cells (Barnes, 1994). On the other hand, we found that synaptic responses mediated by activation of NMDAR were significantly increased both in amplitude and duration in rats pretreated by 192 IgG-saporin as we previously reported with the use of intracellular recordings (Jouveneau et al., 1997). Because the

presynaptic fibre volley was not altered in these animals, this enhanced NMDAR mediated responses might not be due to a sprouting of glutamatergic fibres in response to the loss of cholinergic afferences. In addition, it might result from an impaired activation of cholinergic heteroreceptors presynaptically localized on glutamatergic terminals inhibiting glutamate release (Marchi et al., 1989). However, this hypothesis predicts a parallel increase in non-NMDAR responses and we found that these responses were not affected. Finally, an increase in NMDAR binding sites seemed unlikely because it was reported to be unaffected or even decreased after cholinergic deafferentation by 192 IgG-saporin (Roßner et al., 1995; Nicolle et al., 1997). In view of these results, we suggest that the increase in NMDAR activation induced by the cholinergic denervation may rather reflect changes in the gating properties of associated channels as supported by the significant increase in the duration of these glutamatergic responses (see also Jouveneau et al., 1997). Interestingly, we found that the amplitude of NMDAR mediated responses was not altered in aged animals despite the assumption of fewer synaptic contacts between glutamatergic afferent fibres and pyramidal cells in these animals (see also Billard et al., 1997) suggesting the occurrence of compensatory mechanisms. Considering the increased NMDAR activation recorded in the cholinergic deafferented hippocampus of 192 IgG-saporin pretreated rats, we may hypothesize that the age-related cholinergic deficit may be one possible mechanism of this compensation.

In conclusion, this study shows that in CA1 hippocampal area: i) the glutamatergic neurotransmission is differentially affected by the cholinergic denervation depending on the glutamate subtype of receptors; and ii) the age-related cholinergic deficit may facilitate NMDAR activation and consequently synaptic plasticity in aged animals.

References

- Barnes CA (1994) Normal aging: regionally specific changes in hippocampal synaptic transmission. *TINS* 17, 13–18.
- Barnes CA, Rao G, Foster TC and McNaughton BL (1992) Region-specific age effects on AMPA sensitivity: electrophysiological evidence for loss of synaptic contacts in hippocampal field CA1. *Hippocampus* 2, 457–468.
- Billard JM, Jouveneau A, Lamour Y and Dutar P. NMDA receptor activation in the aged rat: Electrophysiological investigations in the CA1 area of the hippocampal slice *ex vivo*. *Neurobiol. Aging* 1997;18:535–542.
- Giovannini MG, Giovanelli L, Bianchi L, Kalfin R and Pepeu G (1997) Glutamatergic modulation of cortical acetylcholine release in the rat: a combined *in vivo* microdialysis, retrograde tracing and immunohistochemical study. *Europ. J. Neurosci.* 9, 1678–1689.
- Jouveneau A, Billard JM, Lamour Y and Dutar P. Potentiation of glutamatergic EPSPs in rat CA1 hippocampal neurons after selective cholinergic denervation by 192 IgG-saporin. *Synapse* 1997;26:292–300.
- Marchi M, Bocchieri P, Garbarino f and Raiteri M (1989) Muscarinic inhibition of endogenous glutamate release from rat hippocampus synaptosomes. *Neurosci. Lett.* 96, 229–234.
- Markram H and Segal M (1990) Long-lasting facilitation of excitatory postsynaptic potentials in the rat hippocampus by acetylcholine. *J. Physiol (London)* 427, 381–393.
- McEntee WJ and Crook TH (1993) Glutamate: its role in learning, memory, and the aging brain. *Psychopharmacology* 111, 391–401.
- Muir JL (1997) Acetylcholine, aging and Alzheimer's disease. *Pharmacol. Biochem. Beh.* 56,687
- Nicolle MM, Shivers A, Gill TM and Gallagher M (1997) Hippocampal N-Methyl-D-Aspartate and kainate binding in response to entorhinal cortex aspiration or 192 IgG-saporin lesions of the basal forebrain. *Neuroscience* 77,649–659.

- Papathodoropoulos C and Kostopoulos G (1996) Age-related changes in excitability and recurrent inhibition in the rat CA1 hippocampal region. *Europ. J. Neurosci.* 8, 510-520.
- Robner S, Schliebs R and Bigl V (1995) 192 IgG-saporin induced immunotoxic lesions of cholinergic basal forebrain system differentially

affect glutamatergic and GABAergic markers in cortical rat brain regions. *Brain Res.* 696, 165-176.

- Wiley RG, Oeltmann TN and Lappi DA (1991) Immunolesioning: selective destruction of neurons using immunotoxin to rat NGF receptor. *Brain Res.* 562, 149-153.

12

Acetylcholine synthesis and quantal release from mouse neuroblastoma after co-transfection of choline acetyltransferase and mediatophore cDNAs

A. Bloc^a, E. Roulet^a, E. Bugnard^a, F. Loctin^a, J. Falk-Vairant^a, M. Israël^b, Y. Dunant^a

^aPharmacologie, Centre médical universitaire, 1211 Genève 4, Switzerland

^bLaboratoire de Neurobiologie cellulaire et moléculaire, CNRS, 91198 Gif-sur-Yvette, France

The non-cholinergic mouse neuroblastoma cells N18TG-2 do not express proteins such as choline acetyltransferase (ChAT) or vesicular acetylcholine transporter. Even when loaded with acetylcholine (ACh) they are not able to release the neurotransmitter (Falk-Vairant et al., 1996; Israël et al., 1994). They provide therefore an appropriate preparation for investigating the molecular requirements of calcium-dependent ACh release, and its relationship with ACh synthesis.

In the present work, we have studied the effect of N18TG-2 transfection with either rat ChAT cDNA or *Torpedo* mediatophore cDNA or both. The mediatophore is a proteolipid isolated from presynaptic membrane of *Torpedo* cholinergic nerve terminals. ACh release was determined by means of both a chemiluminescent assay and an electrophysiological assay using *Xenopus* myocytes as ACh detectors. When transfected with a plasmid coding for the rat ChAT, N18TG-2 cells accumulate endogenously synthesised ACh but, confirming previous results (Zhong et al., 1995), they are still unable to release the neurotransmitter. In contrast, upon transfection with a plasmid coding for the 16 kDa subunit of the *Torpedo* mediatophore, N18TG-2 acquire the ability to release artificially loaded ACh (Falk-Vairant et al., 1996). When co-transfected with both rat ChAT and *Torpedo* 16 kDa subunit cDNAs, N18TG-2 cells can then release endogenously synthesised ACh.

We further examined whether the release process supported by the mediatophore shows any difference depending on the source of neurotransmitter (loaded or synthesised). Results from real time detection of release using an ACh-sensitive *Xenopus* myocyte show no difference in the kinetic parameters of ACh secretion. The observed time constants are furthermore very close from values recorded in immature *Xenopus* neuro-muscular synapse (Girod et al., 1992). Quantal analysis, based on the failure ratio, also shows no difference in the quantal size, estimated in both cases at about 200 pA, i.e., about 500-1000 ACh molecules, which roughly corresponds to the size of a subminiature potential in the *Torpedo* electric organ or a neuromuscular junction. On the other hand, when the amount of ACh released is expressed as a function of cellular content, it is found that N18TG-2 cells transfected with mediatophore subunit alone and loaded with ACh release only 15.5% of their initial content, whereas this percentage

is about 70% in the case of ChAT/16kDa co-transfected N18TG-2 cells. The co-transfected cells utilise thus a larger proportion of their initial transmitter store; in other words they seem more efficient for release than ACh-loaded cells. Different hypotheses can be put forward to explain this difference: 1) ChAT transfection could indirectly induce the expression of other synaptic proteins (Bignami et al., 1997), which could up-regulate the release process; 2) endogenously-synthesised ACh could be in a better position for fuelling the release process than externally-loaded ACh; and 3) the rate of ChAT activity could be boosted during release, supplying extra amounts of ACh, as previously observed in situ with a variety of preparations.

In conclusion, in N18TG-2 cells the mediatophore can be considered as the minimal molecular requirement for quantal ACh release. This process operates more efficiently when using ACh synthesised by the cells. This increased efficiency of mediatophore-supported ACh release by co-expression of ChAT must have a physiological significance and merits further investigation.

References

- Bignami, F., Bevilacqua, P., Biagioni, S., De Jaco, A., Casamenti, F., Felsani, A. and Augusti-Tocco, G. (1997) Cellular acetylcholine content and neuronal differentiation. *J. Neurochem.* 69, 1374-1381.
- Falk-Vairant, J., Corrèges, P., Eder-Colli, L., Salem, N., Roulet, E., Bloc, A., Meunier, F., Lesbats, B., Loctin, F., Synguelakis, M., Israël, M. and Dunant, Y. (1996) Quantal acetylcholine release induced by mediatophore transfection. *Proc.Natl.Acad.Sci.USA* 93, 5203-5207.
- Girod, R., Eder-Colli, L., Medilanski, J., Dunant, Y., Tabti, N. and Poo, M.M. (1992) Pulsatile release of acetylcholine by nerve terminals (synaptosomes) isolated from the *Torpedo* electric organ. *J. Physiol. Lond.* 450, 325-340.
- Israël, M., Lesbats, B., Synguelakis, M. and Joliot, A. (1994) Acetylcholine accumulation and release by hybrid NG108-15, glioma and neuroblastoma cells - Role of a 16 kDa membrane protein in release. *Neurochem.Int.* 25, 103-109.
- Zhong, Z.G., Misawa, H., Furuya, S., Kimura, Y., Noda, M., Yokoyama, S. and Higashida, H. (1995) Overexpression of choline acetyltransferase reconstitutes discrete acetylcholine release in some but not all synapse formation-defective neuroblastoma cells. *J. Physiol. Paris* 89, 137-145.

13

Downregulation of muscarinic autoreceptor signaling in murine SN56 septal cholinergic cells by acetylcholinesterase inhibitors due to long-term exposure to released acetylcholine

J.K. Blusztajn, R.O. Davis

*Department of Pathology and Laboratory Medicine, Boston University School of Medicine,
85 East Newton Street, Room M1009, Boston, MA 02118, USA*

Acetylcholinesterase (AChE) inhibition elevates acetylcholine (ACh) concentrations within cholinergic neurons and in the synaptic cleft, causing prolonged interaction of ACh with its receptors. We describe the establishment and characterization of an *in vitro* model that resembles the cholinergic synapse, and the application of this model to the study of the effects of AChE inhibitors on the functional properties of the cholinergic synapse. The model employs a murine cholinergic neuronal cell line, SN56, that possesses both the capacity to synthesize, store, and release ACh, as well as to respond to ACh. The cellular responses to ACh are mediated by muscarinic receptors coupled to elevations of intracellular calcium concentrations ($[Ca^{2+}]_i$). Thus, SN56 cells can be used to study both the regulation of ACh synthesis and release as well as the regulation of muscarinic receptor function, i.e., the pre- and the postsynaptic cholinergic mechanisms. We studied the functional sequelae of the exposure of SN56 cells to AChE inhibitors by determining their ACh content and changes in intracellular free calcium concentrations ($\Delta[Ca^{2+}]_i$) in response to a muscarinic agonist, carbachol. Two inhibitors belonging to the class of organophosphorus (OP) compounds, DFP or paraoxon, caused time- and concentration-dependent elevations of intracellular ACh content by as much as nine-fold. The EC_{50} values for these OP were 0.1 μ M and 2.5 μ M, respectively. Similar elevations in ACh content were found in cells treated with the carbamates neostigmine and physostigmine. Moreover, in cells treated with AChE inhibitors, the spontaneously released ACh accumulated in the medium due to inactivation of surface AChE. Basal $[Ca^{2+}]_i$ in SN56 cells was 30–150 nM. Carbachol (1 mM) increased $[Ca^{2+}]_i$ to a maximum of 500 nM within 10 s. This effect was prevented by 1 μ M atropine. In cells treated for 24 h with DFP (10 μ M), paraoxon (10 μ M) or physostigmine (100 μ M), carbachol increased $[Ca^{2+}]_i$ to a maximum of only 250 nM, 120 nM and 160 nM, respectively. The effect of AChE inhibitors required long-term exposure (hours) as a 10-min preincubation of the cells with paraoxon or a 30 s exposure to physostigmine had no effect on carbachol-evoked $\Delta[Ca^{2+}]_i$. To determine if the OP specifically downregulated $\Delta[Ca^{2+}]_i$ evoked by muscarinic receptors, we tested the effects of these OP on bradykinin- and thapsigargin-evoked $\Delta[Ca^{2+}]_i$. The response to bradykinin is mediated by seven transmembrane domain receptors (Farmer and Burch, 1992) similar to muscarinic receptors, whereas thapsigargin causes elevations in $[Ca^{2+}]_i$ by inhibiting the endoplasmic reticulum Ca^{2+} -ATPase (Thastrup et al., 1990). Bradykinin (10 μ M) or thapsigargin (1 μ M) increased $[Ca^{2+}]_i$ in SN56 cells (by 250 and 300 nM, respectively). DFP (10 μ M, 24 h) had no effect on these $\Delta[Ca^{2+}]_i$.

These data can be explained as follows. SN56 cells treated with AChE inhibitors accumulate large amounts of ACh because the newly-synthesized neurotransmitter cannot be hydrolyzed by the inactivated AChE. Some of this ACh is released spontaneously and accumulates in the medium because the membrane-associated AChE is also inactive. ACh in the medium binds to the muscarinic receptors, which desensitize upon long-term exposure to the ago-

nist. As expected, this desensitization is specific for muscarinic receptors, as no desensitization of bradykinin receptors is seen and no changes in general cellular calcium homeostasis occur, as evidenced by the lack of changes in the basal $[Ca^{2+}]_i$ and by the absence of an effect of AChE inhibitors on the cellular responses to thapsigargin. This explanation is consistent with the demonstrations that muscarinic receptors desensitize rapidly upon exposure to an agonist *in vitro* (Tobin et al., 1992; Dell'Acqua et al., 1993; Robbins, 1993; Wojcikiewicz et al., 1994; Inoue et al., 1995; Schmidt et al., 1995; Coggan and Thompson, 1997) and with observations made in rats that treatment with DFP results in reductions of muscarinic receptor number (Schiller, 1979; Schwartz and Kellar, 1983; Aronstam et al., 1987; McDonald et al., 1988; Abdallah and El-Fakahany, 1991; Bushnell et al., 1991) accompanied by cognitive impairments (McDonald et al., 1988; Bushnell et al., 1991). Recent studies have shown that the latter action of DFP involves not only the reduction of muscarinic receptor numbers but also lowering of mRNA expression (Yagle and Costa, 1996).

Our *in vitro* model can be applied to the study of multiple compounds, including potentially toxic substances, that may affect any of the pre- or postsynaptic cholinergic mechanisms, because it permits the evaluation of multiple endpoints in a single experiment. AChE inhibitors are used clinically to treat certain diseases (e.g., the centrally-active AChE inhibitors tacrine or E2020 are used for the treatment of Alzheimer's disease). In addition humans may be exposed to AChE inhibitors (e.g., pesticides or chemical weapons). Indeed there is evidence that Gulf War Syndrome may be the result of exposure to low levels of OP. Recent studies have shown that exposure of rats to low doses of DFP causes impairment of spatial learning (Buccafusco et al., 1997; Prendergast et al., 1997) and downregulation of muscarinic and nicotinic receptors (Buccafusco et al., 1997; Wickelgren, 1997). These *in vivo* results point to a heretofore unappreciated adverse effect of AChE inhibitors: long-term cognitive impairment. The current use of AChE inhibitors (tacrine, E2020) to treat Alzheimer's disease may result in the downregulation of muscarinic and nicotinic receptors in the brains of patients taking these compounds. Receptors in brain areas targeted by the disease process (e.g., hippocampus and cortex) are probably not going to be adversely affected by AChE inhibitors because the cholinergic input to those areas is compromised, and the AChE inhibitors are likely at best to restore ACh levels, rather than to cause hyperphysiological concentrations of the neurotransmitter. However, in brain areas spared by Alzheimer's disease (e.g., striatum), ACh levels would be expected to increase, and result in side effects related to the downregulation of muscarinic receptors. Similarly, components of chemical weapons can be assessed in our model for their ability to downregulate muscarinic receptor function. Finally, the model is well suited as a tool for the development of pharmacological strategies to restore the function of muscarinic receptors downregulated by the use of AChE inhibitors. (Supported by US EPA CR821929-01-0.)

References

- Abdallah EA and El-Fakahany EE (1991) Lack of desensitization of muscarinic receptor-mediated second messenger signals in rat brain upon acute and chronic inhibition of acetylcholinesterase. *J Biochem Toxicol* 6:261–268.
- Aronstam RS, Smith MD and Buccafusco JJ (1987) Clonidine prevents the short-term down regulation of muscarinic receptors in mouse brain induced by the acetylcholinesterase inhibitor soman. *Neurosci Lett* 78: 107–112.
- Buccafusco JJ, Prendergast MA, Pauly JR, Terry AV, Jr., Goldstein BD and Shuster LC (1997) A rat model for gulf-war illness-related selective memory impairment and the loss of hippocampal nicotinic receptors. *Soc Neurosci Abstr* 23:90.19.
- Bushnell PJ, Padilla SS, Ward T, Pope CN and Olszyk VB (1991) Behavioral and neurochemical changes in rats dosed repeatedly with diisopropylfluorophosphate. *J Pharmacol Exp Ther* 256:741–750.
- Coggan JS and Thompson SH (1997) Cholinergic modulation of the Ca^{2+} response to bradykinin in neuroblastoma cells. *Am J Physiol* 273:C612–C617.
- Dell'Acqua ML, Carroll RC and Peralta EG (1993) Transfected m2 muscarinic acetylcholine receptors couple to $G_{\alpha i2}$ and $G_{\alpha i3}$ in Chinese hamster ovary cells. Activation and desensitization of the phospholipase C signaling pathway. *J Biol Chem* 268:5676–5685.
- Farmer SG and Burch RM (1992) Biochemical and molecular pharmacology of kinin receptors. *Annu Rev Pharmacol Toxicol* 32:511–536.
- Inoue R, Sakurai A, Tsuga H, Oishi K and Uchida MK (1995) Carbachol-induced desensitization of rat basophilic leukemia (RBL-2H3) cells transfected with human m3 muscarinic acetylcholine receptors. *Gen Pharmacol* 26:1125–1131.
- McDonald BE, Costa LG and Murphy SD (1988) Spatial memory impairment and central muscarinic receptor loss following prolonged treatment with organophosphates. *Toxicol Lett* 40:42–56.
- Prendergast MA, Terry AV, Jr. and Buccafusco JJ (1997) Chronic, low-level exposure to diisopropylfluorophosphate causes protracted impairment of spatial navigation learning. *Psychopharmacology (Berlin)* 129:183–191.
- Robbins J (1993) Agonist-induced inhibition of inositol-trisphosphate-activated IK (Ca) in NG108-15 neuroblastoma hybrid cells. *Pflügers Arch* 422:364–370.
- Schiller GD (1979) Reduced binding of (3H)-quinuclidinyl benzilate associated with chronically low acetylcholinesterase activity. *Life Sci* 24:1159–1163.
- Schmidt M, Fasselt B, Riimenapp U, Bienek C, Wieland T, Van Koppen CJ and Jakobs KH (1995) Rapid and persistent desensitization of m3 muscarinic acetylcholine receptor-stimulated phospholipase D. *J Biol Chem* 270:19949–19956.
- Schwartz RD and Kellar KJ (1983) Nicotinic cholinergic receptor binding sites in the brain: regulation in vivo. *Science* 220:214–216.
- Thastrup O, Cullen PJ, Drobak BK, Hanley MR and Dawson AP (1990) Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} -ATPase. *Proc Natl Acad Sci U S A* 87:2466–2470.
- Tobin AB, Lambert DG and Nahorski SR (1992) Rapid desensitization of muscarinic m3 receptor-stimulated polyphosphoinositide responses. *Mol Pharmacol* 42:1042–1048.
- Wickelgren I (1997) Rat model for gulf war syndrome? *Science* 278:1404.
- Wojcikiewicz RJ, Tobin AB and Nahorski SR (1994) Muscarinic receptor-mediated inositol 1,4,5-trisphosphate formation in SH-SY5Y neuroblastoma cells is regulated acutely by cytosolic Ca^{2+} and by rapid desensitization. *J Neurochem* 63:177–185.
- Yagle K and Costa LG (1996) Effects of organophosphate exposure on muscarinic acetylcholine receptor subtype mRNA levels in the adult rat. *Neurotoxicology* 17:523–530.

14

Regulation of desensitization by beta subunit in neuronal nAChR

S. Bohler^a, P.-J. Corringer^a, J.-P. Changeux^a, S. Bertrand^b, D. Bertrand^b, S.J. Edelstein^c

^aNeurobiologie Moléculaire, URA, D1284, 25, rue du Dr-Roux, 75724 Paris cedex 15, France

^bDépartement de Physiologie, CMU (Faculté de Médecine), 1211 Geneva 4, Switzerland

^cDépartement de Biochimie, Université de Genève, Geneva, CH1211 Switzerland

The high diversity of neuronal nicotinic receptors generates a wide panel of ligand selectivity and desensitization kinetics.

Desensitization takes place upon long-lasting application of ligand, as a final step in the conformational changes of the molecule. It is a key issue for understanding the prolonged action of ligands and the physiological role of these receptors in nicotine dependence.

In heteropentameric receptors, the structures modulating desensitization may localize on the alpha as well as on the beta subunit. Here we report of molecular determinants carried by the beta sub-

unit. Using a series of chimeras between the two pharmacologically distinct receptors $\alpha 3\beta 2$ and $\alpha 3\beta 4$, we show that regions 1–121 and 129–229 of the N-terminal domain independently regulate the rate of desensitization. Each region can transfer the fast desensitizing rate of $\alpha 3\beta 2$ towards $\alpha 3\beta 4$. Both regions contain photoaffinity labeled residues and are likely to interact with the ligand.

Similarly, these two regions control the sensitivity to agonist cytosine, which selectively activates $\alpha 3\beta 4$.

15

Electrostatic homology modelling of a set of ChE-like neural adhesion proteins identifies a shared 'annular' motif with ChEs.
Structural implications for a cell-recognition role of ChEs

S.A. Botti^{a, b}, C. Felder^a, J.L. Sussman^{a, c}, I. Silman^b

Departments of ^aStructural Biology and ^bNeurobiology, Weizmann Institute of Science, Rehovot, 76100 Israel

^cProtein Data Bank, Brookhaven National Laboratory, Upton, NY 11973, USA

The concept of an electrostatic motif on the surface of biological macromolecules as a definite topographical pattern of electrostatic potentials in 3D space, provides a powerful tool for identification

of functionally important regions on the surface of structurally related macromolecules [1]. We employ this tool to analyze the electrostatic properties of cholinesterases (ChEs) from various

species and show that all these structures reveal a negative external surface potential in an 'annular' area around the entrance to the active-site gorge that becomes more negative as one approaches the rim of the gorge. These potentials are highly correlated among the structures examined, down to a sequence identity as low as 35%, indicating that they are a conserved property of the cholinesterase family [2]. We further suggest that the coincidence between a shared topological arrangement of the macromolecular chain and a conserved electrostatic motif can be used to identify proteins sharing a common recognition mechanism. Using this approach, we identify a functional region common to ChEs and to gliotactin (GLI) [3], neurotactin (NRT) [4], and neuroligin-1 (NL-1) [5], a set of neural cell-adhesion proteins which have been suggested to be structurally related to ChEs due to their high sequence similarity, but lacking the key catalytically active serine [6].

In order to confirm the presence of shared structural motifs and to investigate the possibility that they might display similar electrostatic characteristics to ChEs, we have built homology models of the extracellular domains of GLI, NRT and NL-1 and compared them to the known structures of ChEs and of a series of lipases that have the same degree of sequence identity with ChEs and share the same 3D fold (table I). A quantitative analysis of the surface electric potential in the area around the entrance to the active site gorge for AChE, and in the analogous zone for the ChE-like domain of the adhesion proteins, shows that GLI, NRT and NL are all characterized by a high correlation coefficient with AChE and have an average potential in the 'annular' region very similar to that of AChE; in addition, their potential gradient closely follows that of AChE (figure 1). The same analysis performed on lipases with a ChE-like fold does not reveal the presence of a conserved electrostatic motif. These findings, taken together with the evidence for the involvement of ChEs in cell adhesion independent of their catalytic activity, suggests that the ChE-like domain of GLI, NRT and NL-1 may share a common recognition mechanism or a similar ligand in common with ChEs. Further evidence comes from the recent results of a study in which chimeric constructs of the C-terminal transmembrane domain of NRT fused to the extracellular cholinesterase-like domains of DmAChE or TcAChE, were shown to be endowed with the same

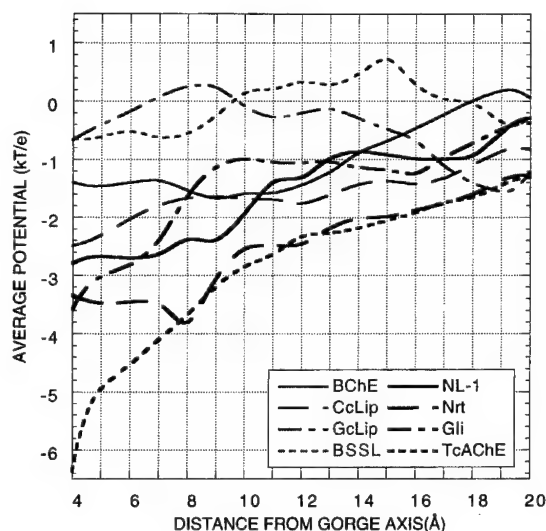


Figure 1.

hydrophilic properties as wild type NRT [7]. On the basis of these findings, we consider GLI, NRT, NL-1 and AChE to be members of a class of adhesion proteins which we have named 'electrotactins'. We hypothesize that a chimeric construct of the cytoplasmic domain of NRT with a mutant form of AChE, in which seven negative residues have been neutralized to abolish the electrostatic motif (7-hAChE) [8], will not display the same adhesive properties as a construct built with wild-type AChE. We further discuss these results in the light of the reported involvement of ChEs in the formation of β -amyloid fibrils [9], the major component of plaques in Alzheimer brain.

Table I.

Protein	Percent identity to TcAChE (DmAChE)	Percent similarity to TcAChE (DmAChE)
<i>Cholinesterases</i>		
TcAChE	100.0	100.0
hAChE	57.6	85.0
mAChE	59.0	85.3
DmAChE	36.3	65.8
7-hAChE	57.6	85.0
hBChE	52.4	83.1
<i>Adhesion proteins</i>		
NRT (Dros.)	31.1 (25.1)	57.7 (50.4)
GLI (Dros.)	30.6 (23.5)	61.0 (55.7)
NL (mouse)	32.3	60.1
<i>Lipases</i>		
GcLip	29.4	56.8
rBSSL	31.2	61.4
CrCE	31.9	56.8

References

- [1] Honig, B. and Nicholls, A., Classical electrostatics in biology and chemistry. *Science* 1995, **268**, 1144-1149.
- [2] Felder, C. E., Botti, S. A., Lifson, S., Silman, I. and Sussman, J. L., External and internal electrostatic potentials of cholinesterase models. *J. Mol. Graphics. Mod.* 1997, **15**, 318-327.
- [3] Auld, V. J., Fetter, R. D., Broadie, K. and Goodman, C. S., Gliotactin, a novel transmembrane protein on peripheral glia, is required to form the blood-nerve barrier in *Drosophila*. *Cell* 1995, **81**, 757-767.
- [4] Barthalay, Y., Hipeau-Jacquotte, R., de la Escalera, S., Jimenez, F. and Piovant, M., *Drosophila* neurotactin mediates heterophilic cell adhesion. *EMBO J* 1990, **9**, 3603-3609.
- [5] Ichtchenko, K., Nguyen, T. and Sudhof, T. C., Structures, alternative splicing, and neuroligin binding of multiple neuroligins. *J. Biol. Chem.* 1996, **271**, 2676-2682.
- [6] Krejci, E., Duval, N., Chatonnet, A., Vincens, P. and Massoulié, J., Cholinesterase-like domains in enzymes and structural proteins: functional and evolutionary relationships and identification of a catalytically essential aspartic acid. *Proc Natl Acad Sci U S A* 1991, **88**, 6647-6651.
- [7] Darboux, I., Barthalay, Y., Piovant, M. and Hipeau-Jacquotte, R., The structure-function relationships in *Drosophila* neurotactin show that cholinesterase domains may have adhesive properties. *EMBO J* 1996, **15**, 4835-4843.

- [8] Shafferman, A., Ordentlich, A., Barak, D., Kronman, C., Ber, R., Bino, T., Ariel, N., Osman, R., Velan, B., Electrostatic attraction by surface charge does not contribute to the catalytic efficiency of acetylcholinesterase. *EMBO J* 1994 13, 3448-3455

- [9] Inestrosa, N. C., Alvarez, A., Perez, C. A., Moreno, R. D., Vicente, M., Linker, C., Casanueva, O. I., Soto, C. and Garrido, J., Acetylcholinesterase accelerates assembly of amyloid-beta-peptides into Alzheimer's fibrils: Possible role of the peripheral site of the enzyme. *Neuron* 1996, 16, 881-891.

16

Biotinylation of the tyrosine residues of fasciculin

P.E. Bougis^a, P. Taylor^b, P. Marchot^a

^aCNRS-UMR 6560, Ingénierie des Protéines, Institut Fédératif de Recherche Jean-Roche, Faculté de Médecine Nord, Université de la Méditerranée, Marseille, France

^bDepartment of Pharmacology, University of California at San Diego, La Jolla, CA, USA

Fasciculins are 7 kDa peptides with four disulfide bridges; they belong to the family of three-fingered snake toxins that includes, among others, the selective nicotinic receptor blockers, α - and κ -neurotoxins [1]. Fasciculins are potent and selective inhibitors of acetylcholinesterase (AChE); they bind to the peripheral site of the enzyme, a site located at the mouth of the structural gorge leading to the buried active center [2]. Kinetic studies have shown that the fasciculin-AChE complex, despite its high affinity and extremely slow rate of dissociation, possesses residual catalytic activity: in the complex in solution, both substrate and catalytic site inhibitors are able to access the active center of the enzyme (cf. [3] for references). However, the recently solved X-ray structure of the Fas2-mouse AChE complex suggested that fasciculin, bound at the peripheral anionic site of the enzyme, totally occludes substrate access to the active site, a departure from the kinetic data [4]. A functional map of the fasciculin molecule was established based on site-directed mutagenesis of several residues located at and near the complex interface [3]; however, the contribution of the tyrosine residues remained to be investigated.

Biotinylation of fasciculins Fas1 and Fas2, which respectively contain four and three tyrosine residues, was carried out using *p*-diazobenzoyl biocytin (DBB). Fas1 was found to be biotinylated on Tyr4, Tyr47 and C-terminal Tyr61, and Fas2, which contains the Tyr47Asn substitution, on Tyr4 and Tyr61. In both cases,

Tyr22 was not reactive, consistent with absence of reactivity toward enzyme-catalysed iodination [5] and non-accessibility to solvent [6]. All the mono-, di- and tri-biotinylated derivatives were characterised structurally and functionally. Their respective inhibitory activities toward mouse AChE were established both kinetically and at equilibrium. Our results complement previous mutagenesis data [3] in providing new insight into the participation of the tyrosine residues of fasciculin in complex formation and stability. This work was supported by the Association Française contre les Myopathies (to PEB and PM).

References

- [1] Cerveñansky' C, Dajas F, Harvey AL & Karlsson E (1991) In *Snake Toxins* (Harvey AL, ed) pp 303-321, Pergamon Press, Inc, NY
- [2] Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Tokor L & Silman I (1991) *Science* 253, 872-879
- [3] Marchot P, Prowse CN, Kanter J, Camp S, Ackermann E, Radic' Z, Bougis PE & Taylor P (1997) *J. Biol. Chem.* 272, 3502-3510
- [4] Bourne Y, Taylor P & Marchot P (1995) *Cell* 83, 503-512
- [5] Marchot P, Khélif A, Ji Y-H, Mansuelle P & Bougis PE (1993) *J. Biol. Chem.* 268, 12458-12467
- [6] Le Du MH, Marchot P, Bougis PE & Fontecilla-Camps JC (1992) *J. Biol. Chem.* 267, 22122-22130

17

Human neuronal nicotinic acetylcholine receptors (nAChRs) may participate in the sexual dimorphism functionality of the brain

B. Buisson, S. Bertrand, D. Bertrand

Department of Physiology, Faculty of Medicine, 1, rue Michel-Servet, CH-1211 Geneva 4, Switzerland

Pathologies such as catamenial epilepsy and postnatal/postmenopausal depressions are associated with modification of women's hormonal status. These paroxysmic situations, which are related to severe variations of circulating sex hormones, indicate that neuronal tissues are influenced by sex hormones (progesterone, 17 β -oestradiol and testosterone). Thus, in non-pathological conditions, it is very likely that sex steroids may influence some of the central nervous system (CNS) functions. As an illustration, a recent positron-emission tomography (PET) study has revealed that progesterone and 17 β -oestradiol modulate cognition-related activities in women¹. From the endocrine point of view, sex steroids are synthesized in gonads (progesterone and 17 β -oestradiol in ovaries, testosterone in testis). However, in the brain testosterone can be converted into 17 β -oestradiol through P450 aromatase activity. To

complicate matters further, some steroids are de novo synthesized by cells of the CNS and constitute endogenous sources of 'neurosteroids'². As a correlate, women and men CNS are exposed to different steroids through their entire life. Thus, it is tempting to speculate that some brain functions may be differentially influenced by sex hormones.

Interestingly, sex differences related to the cholinergic system have been reported. In rodents, a sexual dimorphism of α -bungarotoxin binding in the brain has been observed³. Up-regulation of nAChRs by chronic administration of nicotine has also been shown to be sex related⁴. Functional studies indicate that 17 β -oestradiol is able to potentiate the nicotine-evoked dopamine release in the striatum of female rats whereas it has an opposite effect in the striatum of males⁵. Moreover, the implication of the

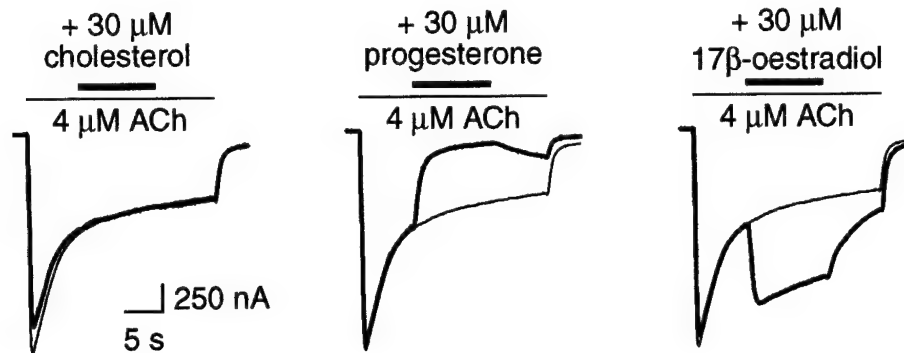


Figure 1. Inhibition and potentiation by progesterone and 17 β -oestradiol of human $\alpha 4\beta 2$ nAChRs expressed in *Xenopus* oocytes.

cholinergic system and especially of neuronal nAChRs is strongly suspected in the sexual differences of rodents for learning task performances such as, for instance, the Morris water maze⁶ or active avoidance paradigms⁷. In man, Alzheimer's disease (AD) affects women more frequently than men⁸.

According to the literature, regulation of CNS functions by sex steroids may implicate two different pathways and time-courses. On one hand, sex steroids induce delayed and long-term effects following their binding to intracellular receptors⁹. On the other hand, these cholesterol-derived molecules have fast-mediated effects on proteins embedded in the cytoplasmic membrane such as G-protein coupled receptors¹⁰, voltage-gated channels (VGCs)^{11, 12} and ligand-gated channels (LGCs)^{13–15}.

Among the vertebrates, neuronal nAChRs constitute a large family of cationic ligand-gated channels (LGCs) encoded by eleven different genes, eight alphas ($\alpha 2$ – $\alpha 9$) and three betas ($\beta 2$ – $\beta 4$) that are broadly distributed within the brain but have also been identified in non-neuronal tissues¹⁶. These receptors result from the assembly of five subunits around a pseudo-axis of symmetry and form both the ionic channel and binding sites for their natural agonist, acetylcholine (ACh). Moreover, neuronal nAChRs present different regulatory binding sites for allosteric modulators such as calcium, arachidonic acid and steroids¹⁷. Although a progressive reduction of neuronal nAChRs is observed during aging, an important loss of nicotinic binding sites was found in patients suffering from AD or Parkinson's disease¹⁸, a reduction that is thought to reflect a specific loss or down regulation of the $\alpha 4$ and/or $\beta 2$ subunits¹⁸. Indeed, the $\alpha 4\beta 2$ nAChR is believed to constitute the nicotine high-affinity binding sites in the brain¹⁹ and to play a central role in smoking addiction^{20–22}. The observation that nicotine as well as other nAChRs ligands can enhance cognitive performances triggered intensive research aiming to identify new molecules that could be of therapeutic use in the treatment of AD or Parkinson's disease²³.

To investigate the influence of sex hormones on human nAChRs, receptors were reconstituted with subunits expressed either in *Xenopus* oocytes or in stably transfected cells. Electrophysiological recordings confirmed that progesterone is a potent negative allosteric modulator of both homomeric and heteromeric nAChRs. In contrast, 17 β -oestradiol at μ M concentration potentiates $\alpha 4\beta 2$ ACh-evoked currents, whereas this steroid still inhibits $\alpha 3\beta 2$ and $\alpha 7$ nAChRs. Macroscopic effects were confirmed at the microscopic level by single channel recordings and demon-

strated that 17 β -oestradiol is a positive allosteric effector at the $\alpha 4\beta 2$ nAChR. These results provide a molecular basis that may explain the lower success of women in smoking cessation trials as well as beneficial effects of estrogen replacement therapy for people suffering from AD. This work was supported by the Swiss National Science Foundation and the OFES to D.B.

References

- Berman, K.F., et al. (1997). *Proc Natl Acad Sci USA* 94, 8836–8841.
- Baulieu, E.E. (1997). *Recent Prog Horm Res* 52, 1–32.
- Arimatsu, Y., Seto, A. & Amano, T. (1981). *Brain Res* 213, 432–437.
- Koylu, E., Demirenen, S., London, E.D. & Pagnier, S. (1997). *Life Sci* 61, 185–190.
- Dluzen, D.E. & Anderson, L.I. (1997). *Neurosci Lett* 230, 140–142.
- Berger-Sweeney, J., Arnold, A., Gabeau, D. & Mills, J. (1995). *Behav Neurosci* 109, 859–873.
- Yilmaz, Ö., Kanit, L., Okur, B.E. & Pöğün, S. (1997). *Behav Pharmacol* 8, 253–260.
- Henderson, V.W. (1997). *Neurology* 48 (suppl 7), S27–S35.
- Jo's, M. (1997). *Front Neuroendocrinol* 18, 2–48.
- Grazzini, E., Guillon, G., Mouillac, B. & Zingg, H.H. (1998). *Nature* 392, 509–512.
- French-Mullen, J.M.H., Danks, P. & Spence, T. (1994). *J Neurosci* 14, 1963–1977.
- Mermelstein, P.G., Becker, J.B. & Surmeier, D.J. (1996). *J Neurosci* 16, 595–504.
- McEwen, B.S. (1996). *Cell Mol Neurobiol* 16, 103–116.
- Lambert, J.J., Bebelli, D., Hill-Venning, C., Callachan, H. & Peters, J.A. (1996). *Cell Mol Neurobiol* 16, 155–174.
- Gu, Q. & Moss, R.L. (1998). *J Physiol (Lond)* 506.3, 745–754.
- Lindstrom, J. (1997). *Mol Neurobiol* 15, 193–222.
- Bertrand, D. & Changeux, J.P. (1995). *Seminars in Neuroscience* 7, 75–90.
- Perry, E.K., et al. (1995). *Neuroscience* 64, 385–95.
- Gopalakrishnan, M., et al. (1996). *J Pharmacol Exp Ther* 276, 289–297.
- Merlo Pich, E., et al. (1997). *Science* 275, 83–86.
- Pidoplichko, V.I., Debiassi, M., Williams, J.T. & Dani, J.A. (1997). *Nature* 390, 401–404.
- Picciotto, M.R., et al. (1998). *Nature* 391, 173–177.
- Newhouse, P.A., Potter, A. & Levin, E.D. (1997). *Drug Aging* 11, 206–228.

and therefore suggests that nAChRs mediating dopamine release also contain these subunits. The subunits still detectable following 6-OHDA-induced lesion may be expressed in non-dopaminergic neurons such as GABAergic neurons and interneurons of the SN and VTA. $\alpha 4$, $\alpha 7$ (expressed only in the SN), $\beta 2$ and $\beta 3$ subunits may be expressed in the large population of GABAergic neurons from the SN pars reticulata which is also sampled together with the SN pars compacta in our experiments, or in GABAergic interneurons present in both the SN and the VTA. However, we cannot exclude the possibility that these subunits are also present on dopaminergic neurons. Indeed, a recent study has demonstrated the probable presence of $\beta 2$ subunit-containing nAChRs in such neurons⁵.

The molecular nature of native nAChRs involved in the modulation of dopaminergic neurotransmission remains controversial^{1,4-7,9} and is the object of considerable interest, particularly in relation to the known addictive properties of nicotine². However, the large number of nAChR subunits expressed in the SN and VTA and the lack of subtype-selective drugs have hampered the characterization of nAChRs responsible for the effect of nicotine on the dopaminergic system. Here, using the RT-PCR methodology, we have identified the different neuronal nicotinic acetylcholine receptor subunit mRNAs expressed selectively in the dopaminergic neurons of the SN and VTA. Our data also pro-

vide direct evidence for the heterogeneity of nicotinic receptor subtypes in dopaminergic neurons. The molecular characterization of these receptors is an essential first step in understanding the relative contribution of different nAChRs in the modulation of physiological processes taking place in midbrain dopaminergic systems.

References

1. Wonnacott S. Trends. Neurosci. 20, 92-98 (1997)
2. Menzaghi F, Whelan KT, Risbrough VB, et al. J. Pharmacol. Exp. Ther. 280, 384-392 (1997)
3. McGehee DS, Role LW. Annu. Rev. Physiol 57, 521-546 (1995)
4. LeNovère N, Zoli M, Changeux JP. European Journal of Neuroscience 8, 2428-2439 (1996)
5. Picciotto MR, Zoli M, Rimondini R, et al. Nature 391, 173-177 (1998)
6. Kaiser SA, Soliakov L, Harvey SC, et al. J. Neurochem. 70, 1069-1076 (1998)
7. Pidoplichko VI, DeBiasi M, Williams JT, Dani JA. Nature 390, 401-404 (1997)
8. Fallon JH, Loughlin SE. Substantia nigra. In: Paxinos G, eds. The rat nervous system. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press, 1995: 215-237
9. Goldner FM, Dineley KT, Patrick JW. Neuroreport. 8, 2739-2742 (1997)

19

Comparison of the effects of mutations and inhibition or activation by cholinergic compounds on kinetic parameters of Acetylcholinesterase: The ESTHER database and server

A. Chatonnet^a, T. Hotelier^b, X. Cousin^a

^aDifférenciation Cellulaire et Croissance and ^bUnité Informatique, INRA-ENSAM, 2, place Viala, 34060 Montpellier, France

ESTHER (for esterases, a/b hydrolase enzyme and relatives) is a database of sequences phylogenetically related to cholinesterases. These sequences define a homogeneous group of enzymes (carboxylesterases, lipases and hormone-sensitive lipases) sharing a similar structure of a central beta-sheet surrounded by alpha-helices (1). The purpose of ESTHER is to provide help with comparing structures and functions of members of the family. In this respect the compilation of compounds acting as inhibitor substrates or reactivators of acetylcholinesterase is valuable to compare kinetic data dispersed in the literature. This is a crucial point when therapeutic effects are sought as some of these compounds can act as inhibitors and slow substrates for cholinesterase (Bambuterol), or act as agonists of acetylcholine receptors and bind acetylcholinesterase as well (Gallamine/flaxedil), or may interfere with choline reuptake and acetylcholine synthesis. Information on kinetic parameters of natural mutants of human butyrylcholinesterase is of utmost importance for monitoring pharmacokinetics of such compounds (2). The database is available through the Internet at: <http://www.ensam.inra.fr/cholinesterase>. The Database Managing System is ACeDB, initially developed for the *C. elegans* genome project (3). Information about this system is available at the USDA server (4). Short tutorials for searching the ESTHER database and building tables have been published (2, 5). Here we present examples of raw data as entered in ESTHER. Knowledge of the different tags or fields available helps formulating queries and building tables.

The maintenance of the system relies on the accuracy of information and up-to-date entering of data. Authors are encouraged to submit data. Example forms are available but any computer-readable files can be sent to us, as the conversion tools used to enter data can be adapted to many different formats. This is made possible by the simplicity of the format of entry of raw data in ACeDB.

References

- (1) Cousin X., Hotelier T., Giles K., Toutant J.-P. and A. Chatonnet (1998) aChEdb: The database system for ESTHER, the a/b fold family of proteins and the Cholinesterase gene server, Nucleic Acids Res., 26 226-228.
- (2) A Chatonnet, T Hotelier, and X Cousin (1998) Kinetic parameters of cholinesterases interactions with organophosphates: retrieval and comparison tools available through ESTHER database. Biol-Chem Interact. in press.
- (3) Durbin R. and Thierry-Mieg J., (1991) A C. elegans Database. Documentation, code and data available from anonymous FTP servers at lirmm.lirmm.fr, cele.mrc-lmb.cam.ac.uk and.ncbi.nlm.nih.gov.
- (4) Matthews D.E. and Sherman B.K., (1996). ACEDB Genome Database Software FAQ, <ftp://rtfm.mit.edu/pub/user-net/news.answers/acedb-faq>, <http://probe.nalusda.gov:8000/acedocs/acedbfaq.html>.
- (5) Chatonnet A., Hotelier T. and Cousin X. (1998) ESTHER (1998), aChEdb a short tutorial, in: B.P. Doctor, D.M. Quinn, R. L. Rotundo, and P. Taylor (Eds.), Structure and Function of Cholinesterases and Related Proteins, Plenum, New York, in press.

20

Relation between AChR and AChE expression and clustering during in vitro neuromuscular synaptogenesis

E. Chaubourt, S. Porte, S. De La Porte

Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS-UPR 9040, Ave de la Terrasse, 91198 Gif-sur-Yvette cedex, France

The formation of the neuromuscular junction (NMJ) requires the clustering of both acetylcholine receptors (AChR) and acetylcholinesterase (AChE), respectively in the postsynaptic membrane and synaptic basal lamina (Hall and Sanes, 1993). We are seeking to elucidate some cellular and molecular mechanisms involved in the expression and clustering of AChR and AChE. Previous studies have suggested that accumulation of AChR is a prerequisite for the formation of AChE clusters (De La Porte et al, 1993, 1998). To clarify the underlying mechanisms we have used two variants of the C2 mouse muscle cell line (Yaffe and Saxel, 1977), S27 and C2R-. C2R- is defective in synthesis of the α -subunit of AChR (Black et al., 1987) and S27 is defective in glycosaminoglycan (GAG) synthesis (Gordon et al., 1993).

C2 wild-type myotubes spontaneously form AChE clusters that are always colocalised with AChR clusters. The S27 myotubes form AChR clusters only in the presence of nerve cells (soluble agrin is not sufficient). In these myotubes we observed no formation of AChE clusters, confirming that the lack of AChR clusters inhibits the accumulation of AChE in the basal lamina.

We analysed the specific activity in both variants. Compared with C2 wild type, the specific activity is increased 4.5-fold in C2R- (deficient in AChR synthesis) and decreased 3-fold in S27 (not deficient in AChR synthesis). This difference suggests that AChE and AChR expressions are related.

We are currently using an antisense strategy in C2 wild type and primary rat myotube culture to study directly the correlation

between AChE and AChR expression and clustering. Preliminary results will be presented.

References

- Hall ZW, Sanes JR (1993) Synaptic structure and development: the Neuromuscular Junction. *Cell* 72/Neuron 10 (suppl.), 99-121
- De La Porte S, Raguet F, Eymard B, Courbin P, Chapron J, Koenig J (1993) Effect of sera from myasthenia gravis patients and of α -bungarotoxin on acetylcholinesterase during in vitro neuromuscular synaptogenesis. *J Neurol Sci* 117, 92-102
- De La Porte S, Chaubourt E, Fabre F, Poulas K, Chapron J, Eymard B, Tzartos S, Koenig J (1998) Accumulation of acetylcholine receptors is a necessary condition for normal accumulation of acetylcholinesterase during in vitro neuromuscular synaptogenesis. *Eur J Neurosci*, in press
- Black R, Goldman D, Hochschwender S, Lindstrom J, Hall ZW (1987). Genetic variants of C2 muscle cells that are defective in synthesis of the α -subunit of the acetylcholine receptor. *J Cell Biol* 105, 1329-1336
- Gordon H, Lupa M, Bowen D, Hall ZW (1993) A muscle cell variant defective in glycosaminoglycan biosynthesis forms nerve-induced but not spontaneous clusters of the acetylcholine receptor and the 43 Kda protein. *J Neurosci* 13 (2), 586-594
- Yaffe D, Saxel O (1977) Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* 270, 725-727

21

Effects of atracurium, a neuromuscular blocking agent, on reconstituted human neuronal nicotinic acetylcholine receptors

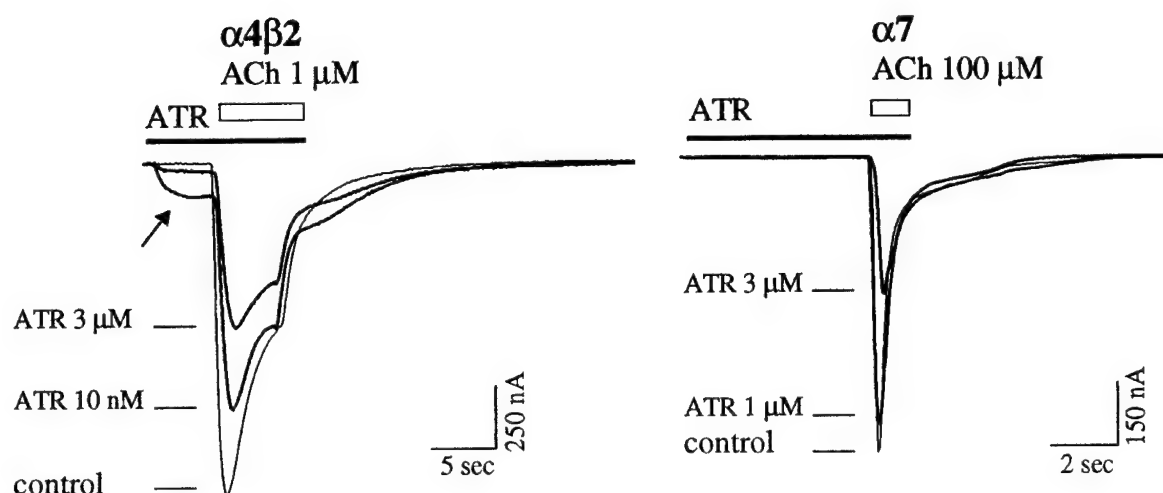
F. Chiodini^a, S. Bertrand^a, E. Tassonyi^b, D. Muller^b, D. Bertrand^a

^aDepartment of Physiology and ^bDepartment of Anaesthesiology, Pharmacology and Surgical Intensive Care, Centre Médical Universitaire de Genève, 1211 Genève 4, Switzerland

(+)-Tubocurarine was the first compound pointing out the existence of a chemical synapse at the neuromuscular junction. It has been extensively documented that (+)-tubocurarine and curaromimetic agents exert several actions¹⁻³ on the nicotinic acetylcholine receptor (nAChR) including: a) a competitive inhibition of the receptor; b) an open channel blockade; and c) in some conditions, an agonist effect. Given their ability to reversibly inhibit transmission at the neuromuscular junction, neuromuscular blocking (NMB) agents are widely used in clinical practice. Although under normal circumstances NMB agents do not cross the blood-brain barrier, detectable amounts of NMB agents and their metabolites can be found in patient's cerebrospinal fluid^{4, 5}. Moreover, we showed that NMB agents modify the synaptic transmission in rat hippocampus. When applied in the nanomolar range, atracurium (ATR) increased the excitatory transmission whereas applications in the micromolar range blocked the inhibitory transmission mediated by the γ -aminobutyric acid⁶. From these results, we hypothesized that NMB agents, and more specifically ATR, can interact with neuronal nAChRs.

To elucidate the mechanism of action of ATR on neuronal nAChRs, we have examined in the present study its effects on human $\alpha 4\beta 2$ and $\alpha 7$ receptors reconstituted in *Xenopus* oocytes. We found that ATR exerts two effects on human receptors. First, ATR reversibly inhibits ACh-evoked currents on both $\alpha 4\beta 2$ and $\alpha 7$ nAChRs with an IC_{50} in the micromolar range (respectively 1.5 and 4.3 μM , see figure 1). To further assess the effect of ATR, the peak of ACh-evoked currents on both $\alpha 4\beta 2$ and $\alpha 7$ receptors was measured at several ACh concentrations in the absence or in the presence of ATR. Application of 10 μM ATR caused a displacement of the dose-response curve toward higher ACh concentrations corresponding to a reduction in apparent affinity, indicating that ATR acts as a competitive antagonist on both receptors. Secondly, when applied alone in the nanomolar range, ATR evoked small but reliable currents in $\alpha 4\beta 2$ receptors (arrow). In contrast, no currents were detected in $\alpha 7$ receptors.

These data illustrate that ATR acts on the human $\alpha 4\beta 2$ and $\alpha 7$ receptors and can inhibit nAChR-mediated neurotransmission



in the central nervous system. As it was shown that neuronal nAChRs can modulate neurotransmission release, it can be proposed that ATR can produce widespread effects⁷⁻⁹. Since NMB agents may reach the central nervous system in patients, these results contribute to the understanding of possible side effects observed in clinical conditions¹⁰. This work was supported by the OFES no 96.0295-1 to D.B. and the Swiss National Science Foundation no 3200.043626.95 to D.M., E.T. and D.B.

References

1. Fletcher GA and Steinbach JH. Ability of nondepolarizing neuromuscular blocking drugs to act as partial agonists at fetal and adult mouse muscle nicotinic receptors. *Mol Pharmacol* 49: 938-947, 1996
2. Liu YI and Dilger JP. Decamethonium is a partial agonist at the nicotinic acetylcholine receptor. *Synapse* 13: 57-62, 1993
3. Trautmann A. Curare can open and block ionic channels associated with cholinergic receptors. *Nature* 298: 272-275, 1982
4. Eddleston JM, Harper NJN, Pollard BJ, Edwards D, Gwinnutt CL. Concentrations of atracurium and laudanosine in cerebrospinal fluid and plasma during intracranial surgery. *Br J Anaesth* 63: 525-530, 1989
5. Tassonyi E, Fuchs-Buder T, Chiodini F, Bertrand D, Muller D and Fathi M. Cerebrospinal fluid concentrations of atracurium in neurosurgical patients (Abstract). *Br J Anaesth* 78: A246, 1997
6. Chiodini FC, Tassonyi E, Fuchs-Buder T, Fathi M, Bertrand D and Muller D. Effects of neuromuscular blocking agents on excitatory transmission and γ -aminobutyric acidA-mediated inhibition in the rat hippocampal slice. *Anesthesiology* 88: 1003-1011, 1998
7. Léna C and Changeux JP. Role of Ca^{2+} ions in nicotinic facilitation of GABA release in mouse thalamus. *J Neurosci* 17 (2): 576-585, 1997
8. Gray R, Rajan AS, Radcliffe KA, Yakehiro M, Dani JA. Hippocampal synaptic transmission enhanced by low concentrations of nicotine. *Nature* 383: 713-716, 1996
9. Guo, J.Z.; Tredway, T.L.; Chiappinelli, V.A. Glutamate and GABA release are enhanced by different subtypes of presynaptic nicotinic receptors in the lateral geniculate nucleus. *J Neurosci* 18 (6): 1963-1969, 1998
10. Miller RD: Muscle relaxants in the ICU. *ASA, Annual Refresher Course Lectures*, San Francisco 255: 1-7, 1994

Morphological alterations of motor nerve terminals after botulinum type-A poisoning or reinnervation of skeletal muscle in the tenascin-C deficient mouse

C. Cifuentes-Diaz^b, F.A. Meunier^a, E. Velasco^b, L. Faille^a, D. Goudou^b, L. Belkadi^b, F. Rieger^b, J. Molgó^a, D. Angaut-Petit^a

^aLaboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, 91198 Gif-sur-Yvette cedex, France

^bLaboratoire de Neuromodulations Interactives et Neuropathologies, INSERM, 17, rue du Fer-à-Moulin, 75005 Paris, France

Tenascin-C is an adhesion molecule of the extracellular matrix involved in many cellular processes during embryogenesis, particularly neurite extension and retraction during development (see review in Chiquet-Ehrismann, 1995). In the adult, tenascin-C is found in myelinating Schwann cells, at the nodes of Ranvier and at the neuromuscular junction (NMJ). In denervated muscles, its expression is up-regulated in endomysial spaces and in perisyn-

naptic interstitial spaces. Its level and distribution return to normal after reinnervation. We have recently shown (Cifuentes-Diaz et al., 1998) that NMJ of tenascin-C deficient mice (Saga et al., 1992) exhibit abnormalities in peripheral nerves and in the NMJ structure. Many abnormalities of Schwann cells and axons were apparent. At the NMJ, preterminal disorganization was prevalent and pre- and post-synaptic abnormalities concerning the number

of axons in a gutter, the number of Schwann cell processes or the aspect of junctional infoldings were observed. Messenger RNA detection by RT-PCR confirmed the presence of low amounts of tenascin-C mRNA levels in skeletal muscle, suggesting that the mice deficient in tenascin-C are not complete knock-outs of this gene, but low-expression mutants.

The aim of the present study was to determine the reactivity and sprouting capability of motor nerves from tenascin-C deficient mice under two experimental conditions: i) after a unique *in vivo* injection of *Clostridium botulinum* type-A toxin (BoTx/A) close to the mouse levator auris longus (LAL) muscle; and ii) after partial denervation of the triangularis sterni (TS) muscle by axotomy of intercostal nerves 3 and/or 4.

The local injection of BoTx/A is known to block acetylcholine release from motor nerve endings causing muscle paralysis and to trigger a profuse sprouting of nerve terminals in normal mice (see Juzans et al., 1996). To determine whether the ability of nerve terminals to expand their field of innervation over the muscle fibers was modified in tenascin-C deficient mice as compared to normal mice, the pattern of motor nerve outgrowth in whole-mount LAL muscles was analyzed and compared 20 days after BoTx/A injection. Sprouting was prominent in LAL muscles from normal mice (figure 1A) and the total nerve terminal length was markedly increased due to an increase in the number of terminal branches and to an increase in average branch length. In contrast, tenascin-C mutant LAL muscles injected with the same dose of BoTx/A exhibited a reduced capability of motor endings to overgrow in response to toxin injection (figure 1B).

At different times after partial denervation of TS muscles, the animals were killed and muscles examined. Marked differences were detected in the reinnervation pattern between tenascin-C mutant and normal muscles. Reinnervation was delayed in the mutant muscles as compared to normals. Furthermore, in each mutant muscle investigated, many motor axons were observed growing beyond their denervated targets, reaching neighboring endplates. As a consequence, many endplates became polyinnervated. Polyinnervation was found to persist for many months after reinnervation (figure 1C), which was never the case in normal mice (see Angaut-Petit et al., 1982).

Many proteins have been suggested to play a role in the formation, maturation and stability of the skeletal neuromuscular junction. Our data, in contrast to recent suggestions by Moscoso et al. (1998), indicate that tenascin-C is involved in nerve terminal outgrowth and in plasticity changes induced by presynaptic perturbations of the NMJ.

References

- Angaut-Petit D, Mallart A, Faille L (1982) Role of denervated sheaths and end-plates in muscle reinnervation by collateral sprouting in the mouse. *Biol Cell* 46, 277-290
- Chiquet-Ehrismann M (1995) Tenascin, a growing family of extracellular matrix proteins. *Experientia* 51, 853-862.
- Cifuentes-Diaz C, Velasco E, Meunier FA, Doudoud D, Belkadi L, Faille L, Murawsky MM, Angaut-Petit D, Molgó J, Schachner M, Saga Y, Aizawa S, Rieger F (1998) The peripheral nerve and the neuromuscular junction are affected in the tenascin-C-deficient mouse. *Cell Mol Biol* 44, 357-379

- Juzans P, Comella JX, Molgó J, Faille L, Angaut-Petit D (1996) Nerve terminal sprouting in botulinum type-A treated mouse levator auris longus muscle. *Neuromuscul Disord* 6, 177-185
- Moscoso LM, Cremer H, Sanes JR (1998) Organization and reorganization of neuromuscular junctions in mice lacking neural cell adhesion molecule, tenascin-C, or fibroblast growth factor-5. *J Neurosci* 18, 1465-1477
- Saga Y, Yagi T, Ikawa Y, Sakakura T, Aizawa S (1992) Mice develop normally without tenascin. *Genes Dev* 6, 1821-1831



Figure 1. Whole mount preparations of LAL (A, B) and TS (C) muscles from normal (A) and tenascin-C mutant mice (B, C) double-stained with an anti-neurofilament antibody and rhodaminated α -bungarotoxin. A, B. The response of nerve terminals 18 days after a unique injection of BoTx/A to a normal (A) and a tenascin-C mutant muscle (B). Note the prominent sprouts in A (arrowheads) and their absence in B. C. A typical example of a reinnervated endplate 7 months after denervation. Notice that two axons supply the same synaptic site. Scale bar, 15 μ m (A); 30 μ m (B); and 7.5 μ m (C).

23 Characterization of mice lacking the nicotinic receptor $\alpha 4$ subunit

L.M. Cordero-Erausquin, L.M. Marubio, M.M.M. Arroyo-Jimenez,
N. LeNovère, M. Huchet, C. Léna, J.P. Changeux

Institut Pasteur, Neurobiologie Moléculaire, 25, rue du Dr-Roux, 75724 Paris cedex 15, France

Nicotine, a drug of addiction, has been shown to have widespread effects in the central nervous system (CNS) by binding to one or more of the numerous subtypes of nicotinic acetylcholine receptors (nAChR). However, the involvement of a particular subunit in pharmacology and behaviour has been difficult to assess. We have made a mutant line of mice by gene targeting and homologous recombination that lacks the neuronal $\alpha 4$ subunit of the nAChR, one of the most widely expressed subunits in the CNS. Mutant mice develop normally, are capable of reproduction, and are indistinguishable from their wild-type littermates in a cage. By *in situ* hybridization, the $\alpha 4$ subunit mRNA is absent in mutant mice, however, no changes in the level of expression of other subunits are observed. ^3H -nicotine, and ^3H -cytisine binding sites are absent in most brain regions of mutant mice, however, some binding does remain in the interpeduncular nucleus, indicating that $\alpha 4$ is involved in most, but not all, of the high-affinity nicotine-binding sites. Furthermore, most ^3H -epibatidine binding sites disappear in mutant mice, however, some binding is still observed in the interpeduncular nucleus, the superior colliculus, the medial habenula, and the substantia nigra. Electrophysiological recordings from brain slices reveal a marked loss of nicotine-induced currents in the thalamus and substantia nigra pars compacta in mutant mice, but currents in the medial habenula are similar to those of wild type mice. These mice will be instrumental in examining the role of high affinity nicotinic receptors in the diverse behavioral effects of nicotine.

ding sites are absent in most brain regions of mutant mice, however, some binding does remain in the interpeduncular nucleus, indicating that $\alpha 4$ is involved in most, but not all, of the high-affinity nicotine-binding sites. Furthermore, most ^3H -epibatidine binding sites disappear in mutant mice, however, some binding is still observed in the interpeduncular nucleus, the superior colliculus, the medial habenula, and the substantia nigra. Electrophysiological recordings from brain slices reveal a marked loss of nicotine-induced currents in the thalamus and substantia nigra pars compacta in mutant mice, but currents in the medial habenula are similar to those of wild type mice. These mice will be instrumental in examining the role of high affinity nicotinic receptors in the diverse behavioral effects of nicotine.

24 Neuropeptidergic facilitation of acetylcholine release from motor nerve terminals depends on tonic A_{2A} -adenosine receptor activation

P. Correia-de-Sá, M.A. Timóteo, J.A. Ribeiro

Lab. Farmacologia, ICBAS, Univ. Porto, and Lab. Neurociências, Fac. Medicina Lisbon, Portugal

It is generally accepted that at the neuromuscular junction co-existing classical neurotransmitters (acetylcholine, ACh) and neuromodulatory peptides (e.g., calcitonin gene-related peptide, CGRP) are released according to a pattern that depends on the stimulation conditions (e.g., Bartfai et al., 1988): neurotransmitter is released alone upon moderate conditions of stimulation, whereas, during high intensity or long term stimulation, neuropeptides are co-released with the classical neurotransmitters. Additionally, stimulation intensity significantly interferes with presynaptic receptor effectiveness (see Duckles and Budai, 1990). Adenosine modulates evoked ACh release by activating both A_1 -inhibitory and A_{2A} -facilitatory receptors. The A_1/A_{2A} -receptor activation balance is highly dependent on the stimulation pattern, e.g., tonic A_{2A} -receptor-mediated facilitation was enhanced when either the stimulus frequency or pulse duration increased (Correia-de-Sá et al., 1996). It therefore seemed of interest to investigate whether activation of the presynaptic A_{2A} -receptor could influence the excitatory effect of two neuromodulatory peptides, CGRP and vasoactive intestinal peptide (VIP), on stimulation (5 Hz, 750 pulses, 0.04 and 1 ms duration) evoked ^3H -ACh release from the rat phrenic motor nerve terminals.

The experiments were performed at 37 °C on rat phrenic nerve-hemidiaphragm preparations loaded with [^3H]-choline (2.5 $\mu\text{Ci/mL}$). The technique used was that described by Wessler and Kilbinger (1986). The preparations were superfused with Tyrode's solution and were continuously gassed with 95% O_2 + 5% CO_2 . The phrenic nerve was electrically stimulated (5 Hz, 750 pulses, 0.04, 1 ms duration) two times at the 12th (S_1) and 39th (S_2) min after the end of the washout period. Test drugs were added 15 min before S_2 and were present up to the end of the experiments. Their effects on transmitter release were expressed by the ratios S_2/S_1 as compared to S_2/S_1 ratio in control experiments.

Both CGRP (100–800 nM) and VIP (30–300 nM) increased evoked ^3H -ACh release in a pulse duration-dependent manner. When 40 μs pulses were used, neither CGRP (200 nM) nor VIP (100 nM) significantly changed evoked transmitter release, however, when stimulus pulse duration increased from 40 μs to 1 ms, both CGRP (200 nM) and VIP (100 nM) facilitated evoked ^3H -ACh release by $36 \pm 4\%$ ($n = 4$) and $64 \pm 33\%$ ($n = 4$), respectively (figure 1). As also shown in figure 1, increases in stimulation pulse duration decreased inhibition of ^3H -ACh release by the nicotinic antagonist, tubocurarine (TC, 1 μM), and favours tonic adenosine A_{2A} (facilitatory) receptor activation. Either inactivating endogenous adenosine, with adenosine deaminase (ADA, 2.5 U/mL), or blocking A_{2A} -receptors, with selective adenosine antagonists, PD 115,199 (25 nM) or DPMX (10 μM), prevented the facilitatory effects of both CGRP (400 nM, $69 \pm 23\%$) and VIP (100 nM, $64 \pm 33\%$). This effect was reversed by pre-incubating the preparations with the non-hydrolysable A_{2A} agonist, CGS 21680C (2 nM). In contrast, no functional interactions between neuropeptides and nicotinic presynaptic receptors were detected.

It has been demonstrated that high intensity motor nerve stimulation: a) potentiates the tonic A_{2A} -receptor mediated facilitation of ACh release, while activation of A_1 -receptors becomes less effective (Correia-de-Sá et al., 1996); b) reduces nicotinic ACh autofacilitation (Wessler, 1989; Correia-de-Sá and Ribeiro, 1994a); and c) increases the magnitude of both CGRP (Correia-de-Sá and Ribeiro, 1994b) and VIP excitation. The present results show that at the rat motor nerve endings presynaptic facilitatory actions of neuropeptides depend on the presence of endogenous adenosine which is tonically activating A_{2A} -receptors. Since both neuropeptides (Kobayashi et al., 1987) and A_{2A} -adenosine (Correia-de-Sá and Ribeiro, 1994c) receptors are positively coupled

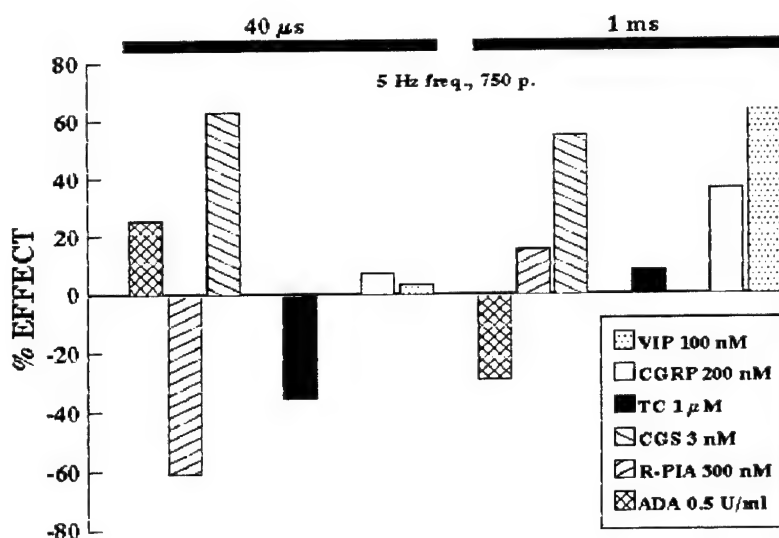


Figure 1. Increases in the stimulation pulse duration: i) decrease nicotinic acetylcholine autofacilitation; ii) potentiate CGRP- and VIP-facilitatory actions; and iii) favour tonic adenosine A_{2A} (facilitatory receptor activation).

to the adenylate cyclase/cyclic AMP system at the rat neuromuscular junction, cooperation between these receptors might occur at the second messenger transduction system level.

References

- Bartfai T, Iverfeldt K, Fisone G, Serfozo P (1988) Regulation of co-existing transmitters. *Annu. Rev. Pharmacol. Toxicol.* 28, 285–310.
- Correia-de-Sá P, Ribeiro JA (1994a) Tonic adenosine A_{2A} receptor activation modulates nicotinic autoreceptor function at the rat neuromuscular junction. *Eur. J. Pharmacol.* 271, 349–355.
- Correia-de-Sá P, Ribeiro JA (1994b) Potentiation by tonic A_{2A}-adenosine receptor activation of CGRP-facilitated ³H-ACh release from rat motor nerve endings. *Br. J. Pharmacol.* 111, 582–588.
- Correia-de-Sá P, Ribeiro JA (1994c) Evidence that the presynaptic A_{2A}-adenosine receptor of the rat motor nerve endings is positively coupled to adenylate cyclase. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 350, 514–522.
- Correia-de-Sá P, Timóteo MA, Ribeiro JA (1996) Presynaptic A₁ inhibitory/A_{2A} facilitatory adenosine receptor activation balance depends on motor nerve stimulation paradigm at the rat hemidiaphragm. *J. Neurophysiol.* 76, 3910–3919.
- Duckles SP, Budai D (1990) Stimulation intensity as critical determinant of presynaptic receptor effectiveness. *Trends Pharmacol. Sci.* 11, 440–443.
- Kobayashi H, Hashimoto K, Uchida S, Sakuma J, Takami K, Tohyama M, Izumi F, Yoshida H (1987) Calcitonin gene-related peptide stimulates adenylate cyclase activity in rat striated muscle. *Experientia* 43, 314–316.
- Wessler I (1989) Control of transmitter release from the rat motor nerve by presynaptic nicotinic and muscarinic autoreceptors. *Trends Pharmacol. Sci.* 10, 110–114.
- Wessler I, Kilbinger H (1986) Release of ³H-acetylcholine from a modified rat phrenic nerve-hemidiaphragm preparation. *Naunyn Schmiedeberg's Arch. Pharmacol.* 334, 357–364.

25

Critical elements determining diversity in agonist binding and desensitization of neuronal nAChR

P.-J. Corringer^a, S. Bertrand^b, S. Bohler^a, S.J. Edelstein^a, D. Bertrand^b, J.-P. Changeux^a

^aNeurobiologie Moléculaire, Institut Pasteur, 25, rue du Docteur-Roux, 75724 Paris cedex 15, France

^bDépartement de Physiologie, Centre Médical Universitaire (Faculté de Médecine), 1211 Geneva 4, Switzerland

Neuronal nicotinic acetylcholine receptors display different pharmacologies according to their subunit composition. In particular, the two putative main classes of brain receptors, $\alpha 4\beta 2$ and $\alpha 7$, display respectively high and low apparent binding and desensitization affinity for agonists, and different specificities for acetylcholine versus nicotine. These features may account for the specific pharmacologies of physiological processes including nicotine addiction, and for the shape of the acetylcholine evoked currents in the brain.

To identify the molecular determinants underlying this diversity, sets of residues from the regions initially identified within the agonist binding site of the $\alpha 4$ subunit were introduced into the $\alpha 7$ agonist binding site, carried by the homooligomeric $\alpha 7$ -V201-5HT3 chimera. Introduction of the $\alpha 4$ residues 183–191 into the $\alpha 7$ -V201-5HT3 selectively increased the apparent affinities for equilibrium binding and for ion channel activation by acetylcholine, resulting in a receptor that no longer displays differences in the responses to acetylcholine and nicotine. Intro-

duction of the $\alpha 4$ residues 151–155 into the $\alpha 7$ -V201-5HT3 produced an approximately 100-fold increase in the apparent affinity for both acetylcholine and nicotine in equilibrium binding measurements. In both cases electrophysiological recordings revealed a much smaller increase (3- to 7-fold) in the apparent affinity for activation, but the concentrations required to desensitize the mutant chimeras parallel the shifts in apparent binding affinity. The data were fitted by a two-state concerted model and an alteration of the conformational isomerization constant leading to

the desensitized state accounts for the 151–155 mutation phenotype, while alteration of the ligand binding site accounts for the 183–191 mutation phenotype.

Point mutation analysis revealed that several residues in both fragments contribute to the phenotypes, with a critical effect of the G152K and T183N mutations. Transfer of $\alpha 4$ amino acids 151–155 and 183–191 into $\alpha 7$ thus confers physiological and pharmacological properties typical of the $\alpha 4\beta 2$ receptor.

26

Dissection of the heparin-binding domains present in the collagenic tail of mammalian acetylcholinesterase

P. Deprez^{a, b}, E. Krejci^b, J. Massoulié^b, N.C. Inestrosa^a

^aUnidad de Neurobiología Molecular, Departamento de Biología Celular y Molecular, P. Universidad Católica de Chile, Chile

^bLaboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS-URA 1857, École Normale Supérieure, 75005 Paris, France

The asymmetric (A) forms of acetylcholinesterase (AChE) consist of one to three catalytic tetramers linked to a collagen-like tail (ColQ), characterized by Gly-X-Y repeats and a high content of imino acids. This collagenic subunit is responsible for the anchorage of the enzyme to the basal lamina, and for its very specific localization at the neuromuscular junction. One of the molecules that could explain the anchorage of the A forms of AChE in the basal lamina is heparan sulfate proteoglycan (HSPG). In this context, competition studies using synthetic peptides led us to describe the presence of two heparin-binding domains (HBDs) in the ColQ subunit of *Torpedo* (Deprez and Inestrosa, 1995). These HBDs are characterized by a central core of basic residues defined by a BBXB motif, and other surrounding basic residues (Cardin and Weintraub, 1989).

Here we present an analysis of the conserved HBDs present on the mammalian ColQ (Krejci et al., 1997) using site-directed mutagenesis. Wild type (wt) and mutated A forms were obtained by co-expression of AChET and rat ColQ mRNA in *Xenopus* oocytes and were then isolated by sucrose gradient velocity se-

dimentation. The relative heparin affinities were evaluated in terms of the concentration of NaCl required for elution of the bound enzyme from heparin-agarose columns using NaCl concentration steps, beginning at 0.2 M and raising the concentration of the next elution step by 0.1 M NaCl.

Using this experimental approach, the wt enzyme presents two main elution peaks, at 0.5 and 0.6 M NaCl (*figure 1*). The first set of ColQ mutations was designed to evaluate the behaviour of each HBD individually. In each case, the core of the domain was altered by replacing a doublet of basic residues with Asp-Pro (DP). We mutated either the N-terminal HBD domain (RKGR DPGR), the C-terminal one (KRGK DPGK) or both (*figure 1*). The double mutant bound heparin poorly, demonstrating that the two identified HBDs are the main heparin-binding sites in ColQ. The enzyme that contains only the N-terminal HBD presents a first peak of elution at 0.3 M NaCl, while the enzyme that only contains the C-terminal HBD first dissociates at 0.4 M NaCl. Interestingly, we were surprised to find that the C-terminal HBD

	<u>N-terminal HBD</u>	<u>C-terminal HBD</u>	<u>First elution peak</u>
WT	GRPGRKGRPGPPGVP	GRPGRKKGQGGQKGDS	0.5 M
KRGK→DPGK	GRPGRKGRPGPPGVP	GRPGDPGKQGGQKGDS	0.3 M
RKGR→DPGR	GRPGDPGRPGPPGVP	GRPGRKKGQGGQKGDS	0.4 M
Double Mutant	GRPGDPGRPGPPGVP	GRPGDPGKQGGQKGDS	0.2 M*
C→N	GRPGKRGKPGPPGVP	GRPGDPGKQGGQKGDS	0.3 M
N→C	GRPGDPGRPGPPGVP	GRPGRKGRQGGQKGDS	0.4 M

Figure 1. Sequence and behaviour of the different mutant enzymes in heparin-agarose columns. For each mutant, the sequences at both the N-terminal and C-terminal domains are shown. In the last row, the NaCl concentration at which each enzyme is eluted is shown. *Only 60% of the double mutant enzyme is bound to the heparin-agarose column.

interacted more strongly with heparin than the N-terminal HBD (figure 1), a fact consistent with the results obtained with triple-helical peptides derived from *Torpedo* ColQ (Deprez and Inestrosa, unpublished results).

To identify the origin of this difference, we replaced the core sequence of the N-terminal HBD by the C-terminal HBD, and vice versa. The results shows that this new C-terminal site (N C) behaves exactly as the wt C-terminal site. Likewise, the new N-terminal HBD (C N) presents the same elution pattern than the wt N-terminal domain (figure 1). These results suggest that the difference between both domains does not depend on the core sequence (KRGK vs. RKGR), but probably on the surrounding residues, that differs on triple-helical stability, as shown by circular dichroism studies (Deprez, Brodsky and Inestrosa, unpu-

blished results). Point mutations on different basic residues are also being presented. This work was supported by FONDECYT No 2970072 to P.D., CNRS and AFM to J.M., and a Presidential Chair in Science from the Chilean Government to N.C.I.

References

- Cardin, AD and Weintraub, HJR (1989) Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis* 9,21-32.
- Deprez, P and Inestrosa, NC (1995) Two heparin-binding domains are present on the collagenic tail of asymmetric acetylcholinesterase. *J. Biol. Chem.* 270, 11043-11046.
- Krejci, E, Thomine, S, Boschetti, N, Legay, C, Sketelj, J and Mas-soulie, J (1997) The mammalian gene of acetylcholinesterase-associated collagen. *J. Biol. Chem.* 272, 22840-22847.

27

Tacrine inhibits L-type calcium channels in the cholinergic SN56 cell line

V. Dolezal, V. Lisá, S. Tucek

Institute of Physiology, Czech Academy of Sciences, Videnská 1083, 142 20 Prague, Czech Republic

The inhibitor of cholinesterases tacrine has recently been introduced into clinical use as a drug for the treatment of Alzheimer's disease but its usefulness is controversial (Byrne and Arie, 1994). In addition to its desired effect, i.e., the preservation of the released acetylcholine, numerous additional effects potentially both beneficial and adverse have been observed in experimental work with tacrine (Freeman and Dawson, 1991; Kelly et al., 1991). We found in our previous work that tacrine and its analog methoxytacrine inhibited the release of acetylcholine from cortical slices and that this effect of both drugs was not related to their anticholinesterase activity (Tucek and Dolezal, 1991; Dolezal and Tucek, 1992). In this study we tested the possibility that tacrine inhibits calcium influx and in this way also the release of acetylcholine.

Using FURA 2 microfluorimetry (Dolezal et al., 1997), we investigated the effect of tacrine on the potassium depolarization-evoked increase of intracellular concentration of free calcium ions ($[Ca^{2+}]_i$) in the SN56 cells derived from mouse septal cholinergic neurons (Hammond et al., 1990). Tacrine inhibited the potassium-evoked increase of $[Ca^{2+}]_i$ with an EC_{50} 3.7 μ M. The inhibition of calcium influx by tacrine was not due to the anticholinesterase activity of the compound. Irreversible inhibitor of cholinesterases paraoxon (10 μ M) had no such effect, and a combination of paraoxon and tacrine decreased the influx of calcium to the same extent as tacrine alone. Nifedipine (1 μ M) inhibited the potassium depolarization induced increase of $[Ca^{2+}]_i$ by 53%, ω -conotoxin GVIA (0.1 μ M) by 39%, and a combination of nifedipine and ω -conotoxin GVIA by 93%. The inhibitory effect of tacrine persisted in the presence of ω -conotoxin GVIA, but not when the L-type calcium channels had been blocked by nifedipine.

Apparently, even low concentrations of tacrine which are relevant to the situation in vivo (Nielsen et al., 1989) may inhibit L-type calcium channels. Many types of calcium channels can serve to carry calcium influx responsible for transmitter release (Turner et al., 1993). Although the electrical stimulation-evoked release of Ach from rat cerebral cortex depends mostly on the influx of calcium through the N-type calcium channels (Dolezal and Tucek, 1987; Wessler et al., 1990) we have shown that the

dependency of transmitter release on the influx of calcium through specific types of calcium channels changes depending on the stimulus used (Dolezal et al., 1996). The inhibitory effect of tacrine on the L-type calcium channels can explain our observation that even 1 μ M tacrine inhibited acetylcholine release from rat cerebrocortical slices evoked by 4-aminopyridine or potassium stimulation. Our data indicate that tacrine should be applied for strengthening of cholinergic transmission with utmost caution. By blocking the L-type calcium channels it can interfere with calcium-dependent events including the release of transmitters. This work was supported by the grant A7011506 of the Grant Agency of the Academy of Sciences of the Czech Republic.

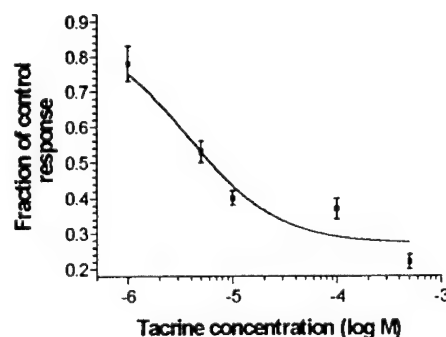


Figure 1. SN56 cells were stimulated twice by 71 mM K^+ , with 20 min between stimulations. The first stimulation was in the absence of tacrine which was added at the indicated concentration (abscissa) 10 min before the second stimulation. The effect of tacrine (ordinate) is expressed as a ratio of peak increase of $[Ca^{2+}]_i$ during the second stimulation and during the first stimulation. Each point represents mean \pm S.E.M. of 41-120 cells from at least two different seedings. Control ratio was 0.883 ± 0.027 ($n = 120$) and the calculated EC_{50} was 3.7 μ mol/L.

References

- Byrne E, Arie T (1994) Tetrahydroaminoacridine and Alzheimer's disease. For the few, but we don't know which few. *Brit. Med. J.* 308, 868-869
- Dolezal V, Tucek S (1987) Failure of the calcium channel activator, Bay K 8644, to increase the release of acetylcholine from nerve terminals in brain and diaphragm. *Br J Pharmacol* 91, 475-479
- Dolezal V, Tucek S (1992) Investigation of the mechanism of the effect of tacrine (tetrahydroaminoacridine) on the metabolism of acetylcholine and choline in brain cortical prisms. *J. Neural. Transm., Park. Dis. Dement. Sect. 4*, 303-318
- Dolezal V, Huang HY, Schobert A, Hertting G (1996) 3,4-diaminopyridine masks the inhibition of noradrenaline release from chick sympathetic neurons via presynaptic $\alpha 2$ -adrenoceptors: insights into the role of N- and L-type calcium channels. *Brain Res.* 721, 101-110
- Dolezal V, Lisá V, Tucek S (1997) Effect of tacrine on intracellular calcium in cholinergic SN56 cell line. *Brain Res.* 769, 219-244
- Freeman SE, Dawson RM (1991) Tacrine: a pharmacological review. *Progress in Neurobiology* 36, 257-277
- Hammond DN, Lee HJ, Tongard JH, Wainer BH (1990) Development and characterization of clonal cell lines derived from septal cholinergic neurons. *Brain Res.* 512, 190-200
- Kelly KM, Gross RA, MacDonald RL (1991) Tetrahydroaminoacridine (THA) reduces voltage-dependent calcium currents in rat sensory neurons. *Neurosci. Lett.* 132, 247-250
- Nielsen JA, Mena EE, Williams IH, Nocerini MR, Liston D (1989) Correlation of brain levels of 9-amino-1,2,3,4-tetrahydroacridine (THA) with neurochemical and behavioral changes. *Eur. J. Pharmacol.* 173, 53-56
- Tucek S, Dolezal V (1991) Negative effects of tacrine (tetrahydroaminoacridine) and methoxytacrine on the metabolism of acetylcholine in brain slices incubated under conditions stimulating neurotransmitter release. *J. Neurochem.* 56, 1216-1221
- Turner TJ, Adams ME, Dunlap K (1993) Multiple Ca^{2+} channel types coexist to regulate synaptosomal neurotransmitter release. *Proc. Natl. Acad. Sci. USA* 90, 9518-9522
- Wessler I, Dooley DJ, Werhand J, Schlemmer F (1990) Differential effects of calcium channel antagonists (ω -conotoxin GVIA, nifedipine, verapamil) on the electrically-evoked release of [^3H]acetylcholine from myenteric plexus, phrenic nerve and neocortex of rats. *N S Arch Pharmacol* 341, 288-294

28

Do central nicotinic and muscarinic cholinergic receptors play differential roles in attentional and episodic memory mechanisms? A study using a new 5-arm maze protocol

T.P. Durkin, C. Beaufort, T. Maviel, L. Leblond

Laboratoire de Neurosciences Comportementales et Cognitives, UMR CNRS 5807, Université de Bordeaux 1, Avenue des Facultés, 33405 Talence, France

Introduction

The hypothesis that central cholinergic neurons play a key role in learning and memory processes remains controversial. Indeed, data from neuropathological investigations of Alzheimer's disease showed that the degree of dementia and recent memory deficits were correlated with the level of degeneration of ascending projections from the magnocellular forebrain cholinergic complex to the neocortex and hippocampus (Collerton, 1986). Attempts to produce an animal model of Alzheimer's disease have thus used more or less extensive and selective lesions of the cholinergic neurons of the magnocellular forebrain complex. It has often been reported that lesions of the septo-hippocampal pathway produces deficits in working memory whereas lesions of the cholinergic projections from the nucleus basalis to the neocortex produce deficits in reference memory (Wenk et al., 1987). However, data from studies using less non-selective lesion procedures have challenged this concept and have led to the proposal (Muir et al., 1994) that the nucleus basalis-cortical cholinergic projections play a more selective role in attentional than in memory processes per se. In order to confront these two hypotheses and to study the roles of nicotinic and muscarinic receptors in cognitive function, we have developed a new 5-arm maze protocol which enables measures of both sustained visual attention (immediate response) and episodic memory (delayed response).

Materials and methods

The 5-arm maze is a hybrid of the 5 choice serial reaction task (Carli et al., 1983) which enables measures of sustained visual

attention and the 8-arm radial maze which enables measures of spatial memory in rodents. The maze comprises a small start-box equipped with a transparent sliding door fixed opposite to 5 goal arms (35 cm long) which radiate in an angle of 120° from one segment of a circular platform (30 cm diameter). The arms, which contain a food pellet tray, can be lit by a 4 W light bulb housed in a PVC roof which covers the arms and closed by a vertical sliding door. Following 5 sessions of habituation during which C57/Bl6 mice ($n = 16$) were allowed to visit each of the 5 lit arms and to consume the food pellets, mice were trained on a task in which, on each trial, only one randomly selected arm among the five (chance level = 20%) is lit and baited. Using four trials per daily session mice attained the criterion of a minimum of 80% correct choices with a choice latency < 5 s on two consecutive sessions in 15-20 sessions. During acquisition of the basic task the arm was lit until the subject chose an arm. In the next phase mice were trained, over 5 further sessions, to the same criterion when the arm was lit for only 2 s. Mice then underwent 4 more sessions in which signal durations of 2, 1 and 0.5 s were randomly intermixed in order to construct a reference curve for attentional performance. To evaluate the roles of nicotinic and muscarinic receptors, respectively, in sustained visual attention mice were submitted to 4 more sessions, using the latter protocol 20 min after an acute i.p. injection of either the nicotinic antagonist, mecamylamine (4 mg/kg), the muscarinic antagonist, scopolamine HCl (0.8 mg/Kg), the combination of these doses of mecamylamine plus scopolamine or saline. A Latin square design was used in which 4 sub-groups of mice received, in rotation, one of the four different treatments on a given session.

Results

Results showed that each of these drug treatments produced significant effects during a period of 1 h following injection. At these doses, mecamlamine produced a moderate decrease in the percentage of correct choices whereas scopolamine, and in particular the combination of scopolamine plus mecamlamine produced highly significant decreases of correct responding which were very close to chance level. The decrease in the level of correct responding was, however, associated with large and significant increases in choice latencies for each of the drug treatments and which was entirely due to the fact that mice remained for long periods in the start-box before initiating a response which was then effected with unchanged running speed. Thus, while controls exhibited a mean choice latency of 2 s, treated mice exhibited choice latencies of around 7 s (mecamlamine), 12 s (scopolamine) and 19 s (mecamlamine plus scopolamine). All drug effects attenuated during the second hour following injection to values which were not significantly different from controls.

Discussion

The 5-arm radial maze protocol which we have developed is a useful test for measuring sustained visual attention performance in mice. In comparison to the 5 choice serial reaction task, which requires several thousand trials for rats to achieve the same criterion, the current test requires only 65–80 trials. Concerning the pharmacological study, the results suggest that nicotinic and muscarinic receptors are implicated in sustained visual attention since both sub-types of cholinergic antagonist produce decreases in the percentage of correct responding. The large concomitant increase in choice latency constitutes however, an obstacle as to an unequivocal conclusion of a selective effect on attention. The fact that the mice do not emit an immediate response entails that response accuracy no longer depends solely on the level of attention and now more heavily engages the neuronal mechanisms respon-

sible for temporary maintenance of the memory trace or response selection and initiation.

Verification of the hypothesis that nicotinic and muscarinic receptors play differential roles in attentional and episodic memory processes will now have to await the outcome of the second phase in which mice will be tested for their ability to effect a delayed response (1 min–1 h). In this case it will be possible not only to replicate the pharmacological experiment using pre-test drug treatments, but also to use the post-acquisition protocol where mice are injected following signal presentation, thus avoiding any interference from drug-induced changes in the level of attention during acquisition and thus to evaluate their episodic memory performance in the same task. This use of the present 5-arm maze and this two-stage experimental strategy for dissociating attentional and episodic memory performance constitutes a powerful research tool for investigating changes in cognitive function which intervene during aging or which are produced in mice which have received a 'knock-out' mutation of genes responsible for the production of nicotinic or muscarinic receptors and will also enable studies permitting evaluation of nicotinic and muscarinic treatment strategies for ameliorating cognitive function.

References

- Carli, M., Robbins, T., Evenden, J.L. and Everitt, B.J. (1983) Effects of lesions to ascending noradrenergic neurones on performance of a 5-choice serial reaction task in rats: implications for theories of dorsal noradrenergic bundle function based on selective attention and arousal. *Behav. Brain Res.* 9, 361–380.
- Collerton, D. (1986) Cholinergic function and intellectual decline in Alzheimer's disease. *Neurosci.* 19, 1–28.
- Muir, J.L., Page, K., Sirinathsinghji, D., Robbins, T.W. and Everitt, B.J. (1993) Excitotoxic lesions of basal forebrain cholinergic neurones: effects on learning, memory and attention. *Behav. Brain Res.* 57, 123–131.
- Wenk, G., Hepler, D. and Olton, D. (1987) Basal forebrain cholinergic neurones and Alzheimer's disease. In *Animal models of dementia, a synaptic neurochemical perspective* (ed Coyle, J.T.) pp187–216; Alan R. Liss New York

29 Elevated acetylcholine release in the hippocampus of transgenic mice expressing the human acetylcholinesterase

C. Erb^a, J. Klein^a, A. Salmon^b, H. Soreq^b, K. Löffelholz^a

^aDepartment of Pharmacology, University of Mainz, 55101 Mainz, Germany

^bDepartment of Biological Chemistry, Life Science Institute, Hebrew University of Jerusalem, Jerusalem, Israel

Introduction

Transgenic mice expressing human acetylcholinesterase (AChE) show an up to two-fold higher AChE activity in various brain regions in comparison to control mice¹. This change in cholinergic balance leads to a progressive impairment of learning and memory which is increasingly significant from the age of 2–3 months on. This learning impairment may be due to a change in cholinergic neurotransmission in the septo-hippocampal pathway which is intimately involved in cognitive processes. Therefore we investigated the level of acetylcholine in the hippocampus using the microdialysis technique.

Materials and methods

An I-shaped microdialysis probe was implanted into the dorsal hippocampus of 5–8-month-old transgenic and control FVB/N mice. On the following 2 days the probe was perfused with artificial cerebrospinal fluid (1 µL/min in neostigmine experiments, 2 µL/min in scopolamine experiments). Samples were collected in 15-min intervals and the concentration of ACh was measured by HPLC with electrochemical detection (BAS), as described previously². The detection limit was 50 fmol. After the experiments we confirmed that the transgenic mice indeed carried the h-AChE transgene by PCR amplification of tail DNA, using primer specific for the human AChE gene.

Results and discussion

The basal hippocampal ACh release was measured in the presence of neostigmine in the perfusion fluid. At low neostigmine concentration (10^{-8} M) the ACh release in transgenic mice was two-fold increased in comparison to the control mice (table I). Differences between transgenic and control mice were not significant at increasing neostigmine concentrations. In extension of previous observations that these mice display resistance to AChE inhibitors as well as enhanced choline uptake³ we conclude that the higher hydrolysis of ACh in transgenic mice is compensated by an increase of the ACh release.

Autoreceptor function in transgenic mice.

The above described increase of the basal ACh release in transgenic mice may be caused by a change of presynaptic muscarinic autoreceptor inhibition. This possibility was tested by studying the responses to scopolamine (1 μ M, in the presence of 10^{-5} M neostigmine) infused via the probe into the hippocampus. The effect of scopolamine on ACh release was similar in transgenic and control mice; the maximum effect of scopolamine in transgenic and control mice appeared after 90 min perfusion with an increase to $693 \pm 127\%$ ($n = 6$) and $592 \pm 95\%$ ($n = 7$) respectively. These results show that in the hippocampus the presynaptic control of ACh release via muscarinic autoreceptors was not changed in transgenic mice.

Table I. Basal efflux of ACh in presence of different neostigmine concentrations.

Neostigmine concentration in the perfusion fluid	ACh efflux [fmol/min]	
	Control mice (n = 4)	Transgenic mice (n = 5)
10^{-8} M	4.5 ± 0.76	$9.8 \pm 0.48^{**}$
10^{-7} M	16.9 ± 2.9	19.4 ± 3.1
10^{-6} M	45.4 ± 4.0	51.8 ± 7.7

$^{**}P < 0.01$.

References

- 1 Beeri R., Andres C., Lev-Lehman E., Timberg R., Huberman T., Shani M. and Soreq H. (1995) Transgenic expression of human acetylcholinesterase induces progressive cognitive deterioration in mice. *Curr. Biol.* 5: 1063–1071.
- 2 Köppen A., Klein J., Erb C. and Löffelholz K. (1997) Acetylcholine release and choline availability in rat hippocampus: effects of exogenous choline and nicotinamide. *J. Pharmacol. Exp. Ther.* 282: 1139–1145.
- 3 Beeri R., Le Novère N., Mervis R., Huberman T., Grauer E., Changeux J. P. and Soreq H. (1997) Enhanced hemicholinium binding and attenuated dendrite branching in cognitively impaired acetylcholinesterase-transgenic mice. *J. Neurochem.* 69: 2441–2451.

30

Regulation of human choline acetyltransferase gene by inhibitors of histone deacetylase

E. Espinos, M.J. Weber

Laboratoire de Biologie Moléculaire Eucaryote, CNRS, 118, route de Narbonne, 31062 Toulouse cedex, France

Choline acetyltransferase (ChAT) activity in sympathetic neuron cultures is increased up to 8-fold by a 2-day treatment with sodium butyrate (5 mM) or with trichostatin (1 μ M) and trapoxin (30 nM), two highly specific inhibitors of histone deacetylase (HDAC) activity. Transfection of CHP126 human neuroepithelioma cells with ChAT-luciferase hybrid genes showed that the M promoter, but not R promoter, is activated up to 100-fold by HDAC inhibitors (Chireux et al., 1994, 1996). Transcriptional activation does not require ongoing protein synthesis, suggesting that HDAC inhibitors activate preexisting transcription factors, possibly through phosphorylation.

The stimulation of M promoter by butyrate and trapoxin is blocked by dominant negative mutants of ras and ERK2, and by PD980589, a highly specific inhibitor of MEK1. Constitutively active mutants of ras and MEK1 have little effects by themselves, but potentiate the effects of butyrate. Therefore, the functioning of the ERK/MEK MAP kinase cascade is required for the activation of ChAT M promoter by HDAC inhibitors (Espinosa and Weber, 1998). In addition, H7, a serine/threonine protein kinase inhibitor, totally blocks the activation of ChAT promoter by butyrate or trapoxin, in both transient and stable transfection assay.

CBP/p300 protein is a co-activator for several transcription factors and display intrinsic histone acetylase transferase (HAT) activity. The overexpression of the p300 stimulates ChAT M pro-

motor, and strongly synergizes with butyrate. The effects of p300 are suppressed by H7, as well as by adenovirus E1A protein.

These data thus show that transcription from the M promoter of human ChAT gene is modulated by the level of acetylation of nucleosomal core histones, and/or other chromatin associated proteins. HDAC inhibitors like butyrate and trapoxin acts by unmasking multiprotein complexes with HAT activity. Histone hyperacetylation is not sufficient for maximal transcription, that requires in addition the functioning of the MAP kinase cascade and of a H7-sensitive serine/threonine kinase.

Small permeant molecules capable of stimulating the transcription of human ChAT gene might help in stimulating acetylcholine synthesis in diseases characterized by cholinergic deficits.

References

- Chireux M, Le Van Thai A, Weber MJ (1994) Human choline acetyltransferase gene: localization of alternative first exons. *J. Neurosci. Res.* 40, 427–438
- Chireux M, Espinos E, Bloch S, Yoshida M, Weber MJ (1996) Histone hyperacetylating agents stimulate promoter activity of human choline acetyltransferase gene in transfection experiment. *Mol. Brain Res.* 39, 68–78
- Espinosa E, Weber MJ (1998) Activation of the MAP kinase cascade by histone deacetylase inhibitors is required for the stimulation of choline acetyltransferase gene promoter. *Mol. Brain Res.* (in press).

31 Two novel α -conotoxins isolated from the venom of *Conus consor*: isolation, synthesis and binding on the *Torpedo* nicotinic acetylcholine receptor

P. Favreau^a, F. Legall^a, D. Servent^b, A. Menez^b, J. Molgo^c, Y. Letourneux^a

^aLaboratoire SESNAB, Université La Rochelle, Pôle Sciences, Av. Marillac, 17042 La Rochelle

^bDIEP, CEA, 91191 Gif-sur-Yvette cedex

^cLaboratoire NBCM, UPR CNRS 9042, 1, Av. de la Terrasse, 91198 Gif-sur-Yvette cedex, France

Venom of the Conidae family is a major source of neuroactive compounds. Several classes of conotoxins have been characterized including α -, μ -, ω -, δ - and κ -conotoxins (Olivera et al., 1990). α -Conotoxins are the smallest conotoxins isolated from the venom of conus, comprising 12 to 19 amino acids with two or three disulfide bonds. This family of paralytic toxins target the nicotinic acetylcholine receptor. Two novel α -conotoxins, α -CnIA and α -CnIB, were isolated from the venom of *Conus consor* and characterized by binding experiments.

Conotoxin α -CnIA is a 14 amino acid peptide showing high homology with conotoxin MI (McIntosh, 1982). α -CnIA inhibits the fixation of [¹²⁵I] α -bungarotoxin on the *Torpedo* nicotinic acetylcholine receptor with an IC₅₀ of 0.7 μ M (figure 1). Binding experiments on the neuronal α 7 receptor indicate no competition up to 10 μ M.

Chemical synthesis of conotoxin α -CnIA was carried out using the Fmoc strategy with selective protection of the four cystine residues (Kamber et al., 1980). This led to correctly folded and biologically active product in reasonable amounts. Synthetic conotoxin compete with [¹²⁵I] α -bungarotoxin with no difference in binding affinity compared to natural toxin.

Synthetic α -CnIA was used for electrophysiological experiments. The toxin progressively diminishes the amplitude of evoked synaptic potentials in frog neuro-muscular junction (cutaneous pectoris). At 1 μ M, complete blockade of the neuro-muscular junction occurs in 25 min. Concomitant disparition of spontaneous miniature end plate potentials clearly shows the post-synaptic effect of the toxin.

These new α -conotoxins are related to a large set of α -conotoxins including conotoxins GI, MI and SI. Thus, they provide novel probes for investigating further the functionality of the nicotinic receptor (Myers, 1991) and the synapse mechanisms.

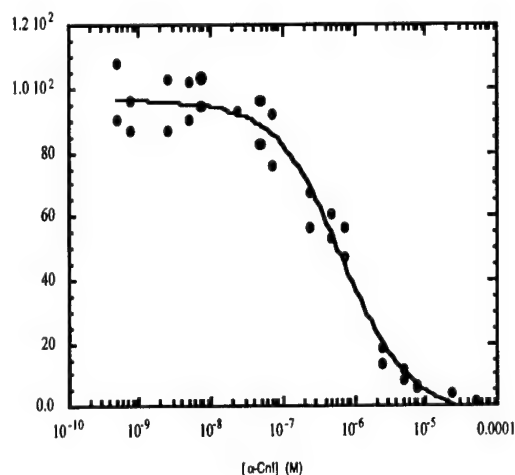


Figure 1. Inhibition of [¹²⁵I]- α -Bgtx binding to nicotinic receptor (*Torpedo* organ) by α -conotoxin CnIA.

References

- Kamber B, Hartmann A, Eisler K, Riniker B, Rink H, Sieber P, Rittel W (1980) The synthesis of cystine peptides by iodine oxidation of S-trityl-cysteine and S-acetamidomethyl-cysteine peptides. *Helv.Chim.Acta* 63, 899-915.
- McIntosh M, Cruz LJ, Hunkapiller MW, Gray WR and Olivera BM (1982) Isolation and structure of a peptide toxin from the marine snail *Conus magus*. *Arch.Biochem.biophys.*, 218, 329-334.
- Myers RA, Zafaralla GC, Gray WR, Abbott J, Cruz LJ, Olivera BM (1991) α -conotoxins, small peptide probes of nicotinic acetylcholine receptors. *Biochemistry*, 30, 9370-9377.
- Olivera BM, Rivier J, Clark C, Ramilo CA, Corpuz GP, Abogadie FC, Mena EE, Woodward SR, Hillyard DR, Cruz LJ (1990) Diversity of *Conus* neuropeptides. *Science* 249, 257-263.

32

Muscarinic receptor-activated arachidonic acid release is enhanced by cytokines in A2058 human melanoma cells

C.C. Felder^a, J.H. Joon^b, E.M. Briley^b, M.W. Wood^a

^aNeuroscience Discovery, Eli Lilly Research Laboratories, Indianapolis, IN 46285, USA

^bLab of Cellular and Molecular Regulation, National Institute of Mental Health, Bethesda, MD 20892, USA

TNF α is a pleiotropic cytokine primarily secreted by activated and resident macrophages such as Kupffer cells and microglia (reviewed in 1). TNF α mediates a wide spectrum of biological activity including cell death, cell proliferation, and lymphocyte activation. Two receptors for TNF α have been identified, the p55 and p75, both of which have been cloned and sequenced. Most mammalian cells express at least one of these receptors. Previous studies have shown that TNF α can stimulate the release of arachidonic acid presumably through activation of phospholipase A2 (2). Neural degeneration of cholinergic neurons such as occurs during Alzheimer's disease, may involve cytokine-mediated inflammatory processes. A 85-kDa phospholipase A2, cPLA2, has been implicated in ischaemic neural loss (3) and is elevated in Alzheimer's brains (4). We investigated possible relationships between cytokine and muscarinic receptor signal transduction mediated by phospholipase A2.

We have previously demonstrated that the human melanoma cell line A2058 expresses only the m5 subtype of muscarinic receptor (5). In A2058 cells, treatment with the cholinergic agonist, carbachol (CC) induces a 2–4-fold increase in arachidonic acid release over basal levels. CC-induced arachidonic acid release is blocked by the muscarinic antagonist, atropine. The CC-induced arachidonic acid release is potentiated 2–3-fold by pretreatment of A2058 cells with either of the inflammatory cytokines, tumor necrosis factor- α (TNF α) or interleukin 1- β (IL-1 β). Enhancement of CC-induced arachidonic acid release by TNF α pretreatment peaks near 1 h, consistent with a transcriptionally derived event. Western analysis of I κ B α , the inhibitory subunit of the NF κ B complex, suggests that both cytokines are capable of activating κ B-driven transcription. Pretreatment of A2058 cells with either of the proteasome inhibitors, MG-115 or MG-132, blocks

the cytokine-dependent degradation of I κ B α but does not affect the enhancement of CC-induced arachidonic acid release. Western analysis demonstrates that both cytokines can trigger the phosphorylation (and activation) of p38 MAP kinase. Furthermore, pretreatment of A2058 cells with the p38 kinase inhibitor, SB202190, ablates cytokine-dependent augmentation without interfering with CC-induced AA release. Finally, no change in the phosphorylation state of the p44/42 MAP kinases follows cytokine treatment. Thus, inflammatory cytokines may regulate muscarinic signaling through the p38 MAP kinase pathway. Additionally, this study provides evidence that potentiation of muscarinic receptor-mediated signal transduction may be a direct result of inflammation.

References

- Vandenabeele P., Declercq W., Beyaert R., and Fiers W. Two tumor necrosis factor receptors: structure and function. *Trends in Cell Biol* 5:392–399 (1995).
- Suffys P., Beyaert R., De Valck D., Vanhaesebroeck B., Van Roy F., Fiers W. Tumour-necrosis-factor-mediated cytotoxicity is correlated with phospholipase-A2 activity, but not with arachidonic acid release per se. *Europ J Biochem* 195:465–75 (1991).
- Bonventre J.V., Huang Z., Taheri M.R., O'Leary E., Li E., Moskowitz M.A., Sapirstein A. Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A2. *Nature* 390:622–625 (1997).
- Stephenson D.T., Lemere C.A., Selkoe D.J., Clemens J.A. Cytosolic phospholipase A2 (cPLA2) immunoreactivity is elevated in Alzheimer's disease brain. *Neurobiol of Disease* 3:51–63 (1996).
- Kohn E.C., Alessandro R., Probst J., Jacobs W., Briley E.M., Felder C.C. Identification and molecular characterization of a m5 muscarinic receptor in A2058 human melanoma cells: Coupling to inhibition of adenylate cyclase and stimulation of phospholipase A2. *J Biol Chem* 271:17476–17484 (1996).

33

Modulation of dopamine release by the nicotinic agonist epibatidine in the frontal cortex and the nucleus accumbens of naive and chronic nicotine treated rats

L. Friberg, I. Bednar, A. Nordberg

Department of Clinical Neuroscience and Family Medicine, Division of Molecular Neuropharmacology, Karolinska Institute, Huddinge University Hospital, B84, S-141 86 Huddinge, Sweden

Accumulating data indicate that the neuronal nicotinic receptors (nAChR) are involved in memory, learning and dependence processes in brain (Newhouse, 1997; Lindstrom, 1997). The reduced number of nAChRs in neurodegenerative disorders, such as Alzheimer's disease (Nordberg, 1992), motivates further research to understand the properties of the various nAChRs, their distribution, physiological function and pharmacological properties.

The nAChRs belong to the family of ligand-gated ion channel receptors. The nAChRs consist of two type of homologous α and β subunits. To date, eight α (α 2– α 9) and three β (β 2– β 4) subunits have been cloned which can appear in different combinations (Gotti et al., 1997) with probably different functional properties.

Three nAChRs binding sites have been found in brain using nicotinic receptor ligands such as nicotine, epibatidine and α -bungarotoxin with different affinities to α 4, α 3, α 7 nAChRs subunits.

Interactive mechanisms between the cholinergic and dopaminergic systems seems to be important in learning, memory and dependence processes in which DA is suggested to play a role in attention and reward mechanisms (Wise, 1978; Beninger, 1983).

Nicotine has been shown to stimulate dopamine release in brain (Wonnacott, 1997). The involvement of nAChRs is not fully understood although both α 4, α 3 nAChRs have been suggested to be involved.

In this study we used the microdialysis technique to investigate the effect of the nicotinic agonist epibatidine (2.5 µg/kg subcutaneous) on nAChR-mediated dopamine release in the frontal cortex and nucleus accumbens of freely moving rats. We found that epibatidine significantly reduced the release of dopamine in both the nucleus accumbens and in the frontal cortex. The effect on DA release was more pronounced in the nucleus accumbens compared to the frontal cortex. A single dose of epibatidine also caused a significant increase in DOPAC levels in the frontal cortex while no effect was observed in the nucleus accumbens. In addition, the HVA levels were significantly increased in both brain regions. Pretreatment with the nicotinic antagonist mecamylamine (2 mg/kg) partly inhibited the epibatidine-induced decreased release of dopamine in the nucleus accumbens. The findings suggest an opposite effect of epibatidine compared to nicotine on dopamine release in nucleus accumbens and frontal cortex. The finding suggests that different nAChR subtypes may mediate the effect of nicotine and epibatidine on dopamine release respectively.

Chronic treatment with nicotine is known to upregulate the nAChRs in brain. We have earlier observed that the nAChRs differ in their magnitude of up-regulation and that the $\alpha 4 \beta 2$ nAChR is more readily up-regulated than the $\alpha 3$ nAChRs subunit (Warpman et al., 1998). Nisell et al. (1996) found that subchronic nicotine treatment enhanced the effect seen by nicotine on dopamine release in the frontal cortex but not in the nucleus accumbens. Marshall et al. (1997) however recently reported no significant effect of nicotine on dopamine release in the frontal cortex of rats subchronically treated with nicotine. In the present study we found that treatment with nicotine (0.45 mg/kg base s.c. twice daily) for 7 days counteracted the decrease in dopamine release seen following a single dose of epibatidine while no effect of subchronic nicotine treatment was observed in the frontal cortex. The

findings suggest changes in nAChR subtype properties following repeated nicotine treatment which may change the epibatidine-induced dopamine release. The studies are expected to provide further insight into the modulatory effect of nicotinic receptor on dopamine release in different brain regions and the particular nAChRs subtypes involved in these specific processes.

References

- Newhouse P, Potter A, Levin E (1997) Nicotinic system involvement in Alzheimer's and Parkinson's Diseases. *Drug & Aging* 11 (3), 206-228
- Lindstrom J (1997) Nicotinic Acetylcholine Receptors in Health and Disease. *Molecular Neurobiology*, 15 (2), 193-222
- Nordberg A (1992) Neuroreceptor changes in Alzheimer's disease. *Cereb. Brain. Met. Rev.* 4, 303-328
- Gotti C, Fornasari D, Clementi F (1997) Human neuronal nicotinic receptors. *Progr in Neurobiol.* 199, 199-237
- Wise R. A (1978) Catecholamine theories of reward: A critical review. *Brain Res.* 152, 215-247
- Beninger R. J (1983) The role of DA in locomotor activity and learning. *Brain Res.* 6, 173-196
- Wonnacott S (1997) Presynaptic nicotinic ACh receptors. *Trends Neurosci.* 20, 92-98
- Warpman U, Friberg L, Gillespie A, Hellström-Lindahl E, Zhang X, Nordberg A (1998) Regulation of nicotinic receptor subtypes following chronic nicotinic agonist exposure in M10 and SH-SY5Y neuroblastoma cells. *J. Neurochem.* 70, 2028-2037
- Nisell M, Nomikos G, Hertel P, Panagis G, Svensson T (1996) Condition-independent sensitization of locomotor stimulation and mesocortical dopamine release following chronic nicotine treatment in the rat. *Synapse* 22, 369-381
- Marshall DL, Redfern PH, Wonnacott S (1997) Presynaptic nicotine modulation of dopamine release in the three ascending pathways studied by in vivo microdialysis: comparison of naive and chronic-treated rats. *J Neurochem* 68: 1511-1519.

34

The segregation of a lipid domain underlies structural and functional modulation of acetylcholine receptor in reconstituted membranes

J.M. González-Ros, A.M. Fernández, J.A. Encinar, J.A. Poveda

Centro de Biología Molecular y Celular, Universidad Miguel Hernández. 03206 Elche (Alicante), Spain

Purified acetylcholine receptor (AChR) from *Torpedo* has been reconstituted into whole asolectin lipids or into defined multi-component liposomes made of 50% phosphatidylcholine, 25% cholesterol plus 25% of one of the following phospholipids: phosphatidylcholine (PC), phosphatidyl-ethanolamine (PE), phosphatidylglycerol (PG) or phosphatidic acid (PA) (Avanti polar lipids). These phospholipids were all derivatives of egg PC and therefore, have the same fatty acid composition.

The ability of the reconstituted samples to promote cation translocation in response to agonist (carbamylcholine) binding was assessed by using a 'stopped-flow /fluorescence quenching' assay of TI^+ influx. As expected from previously published data in similar lipid media, the samples reconstituted in vesicles made exclusively from zwitterionic PC/cholesterol mixtures completely lacked ion channel activity. On the contrary, the presence in the vesicles of phospholipids other than PC retains AChR activity to an extent which varies depending upon the phospholipid. Thus, the largest responses to cholinergic agonists observed correspond to samples containing PA (about one half of the maximal response seen in the samples reconstituted in whole asolectin, used as a

reference for full functional reconstitution), followed by those containing PG, the other anionic phospholipid used in these studies. Finally, the samples containing the zwitterionic PE exhibited a very low level of cation channel activity.

We are reporting here that specific alterations of the AChR secondary structure accompany the reported lipid-induced effects on AChR function. Fourier-transform infrared (FT-IR) studies of the reconstituted samples show that the amide I band in the infrared spectrum of the protein, when reconstituted in the more active, PA-containing matrix, resembles closely that observed in the fully-functional samples reconstituted in whole asolectin lipids, while it differed considerably from spectra obtained from inactive samples reconstituted in PC/cholesterol mixtures. Analysis of the overlapping secondary structure spectral components in the amide I band contour (Echabe et al., 1997) revealed that the maintenance of AChR function by phospholipids such as PA, can be correlated with the preservation of a high percentage of α -helical components in the protein structure. On the other hand, the absence of such relevant lipids results in inactive reconstituted samples, in which the AChR protein exhibits a significant loss

of α -helical structure and an increase in non-ordered structural components (figure 1).

The question remains as to determining: i) what are the molecular events responsible for the effects of phospholipids in modulating AcChR structure and function?; and ii) why is PA the most effective phospholipid in this regard? In the former, the observation that lipid effects on AcChR structure and function occur correlatively and involve precisely lipids such as PA, with a higher affinity for binding to the AcChR protein (Fong and McNamee, 1987), suggests that the interaction of the lipids with the transmembrane portion of the AcChR is what causes that the lipid-bound protein adopts a functionally-competent structure, capable to respond properly to the agonists in the opening of the AcChR ion channel. To test such a hypothesis in terms of the involvement of possible changes in lipid organization and dynamics, we prepared samples of AcChR reconstituted in lipid mixtures containing 50% egg PC, 25% cholesterol and 25% of either perdeuterated DMPA (d-DMPA) or perdeuterated DMPC (d-

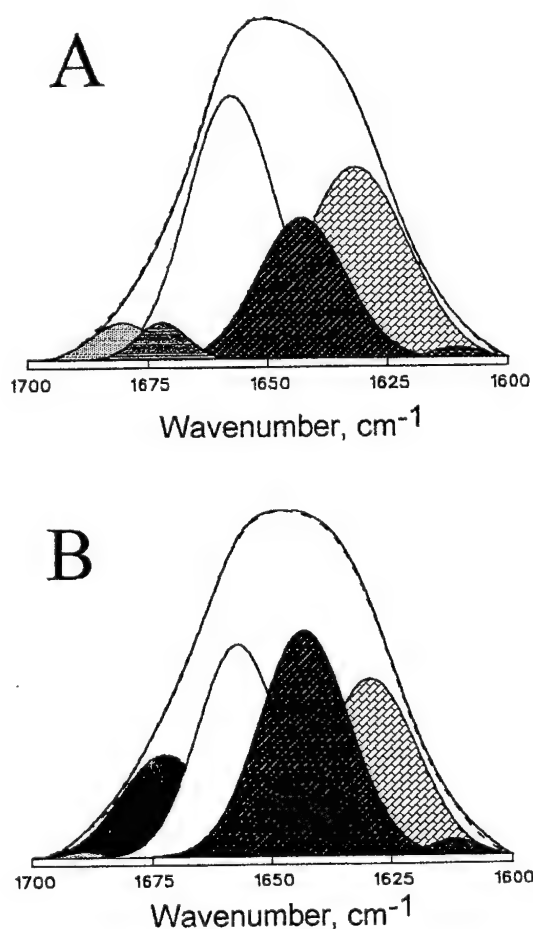


Figure 1. Secondary structure determination from curve-fitting the amide I band of 'functional' (PA + PC + Cho, A) and 'non-functional' (PC + Chol, B) reconstituted samples. Spectral components at 1652 and 1640 cm^{-1} are assigned to α -helical and non-ordered structures, respectively.

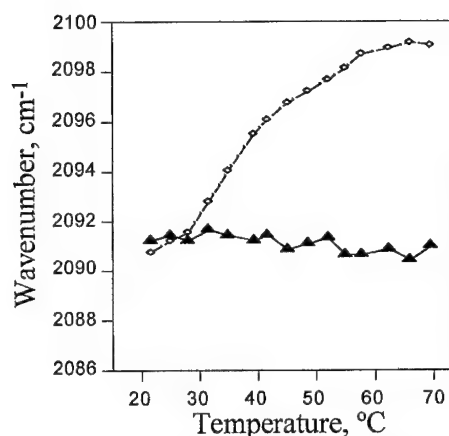


Figure 2. The AcChR protein induces temperature-dependent changes of the CD2 symmetric stretching band of vesicles made from 50% PC, 25% cholesterol and 25% of perdeuterated d-DMPA (diamonds). On the contrary, no temperature-dependent changes can be observed in the absence of protein, suggesting that under conditions, the lipid components are ideally mixed.

DMPC). Such dimyristoyl derivatives were chosen for these studies because of their convenient phase transition temperatures. FT-IR spectroscopy and differential scanning calorimetric (DSC) studies on the effects of the protein on phospholipid dynamics within these reconstituted samples show that in the absence of protein, the complex population of membrane lipids remains ideally mixed. On the contrary, the presence of the AcChR protein in the reconstituted vesicles directs the formation of specific lipid domains that become segregated from the bulk lipid matrix (figure 2). Furthermore, additional FT-IR and DSC studies indicate that such lipid domains are likely to be composed of monoanionic PA and cholesterol.

As to the selectivity exhibited by PA in this process, it has been reported that it can not be accounted for based on electrostatic interaction with the protein (Raines and Miller, 1993). This, along with the fact that all the phospholipids used in this work have identical acyl moieties, lead to the conclusion that the main contributing factor in the interaction with the lipids should be the recognition of the entire polar head group by the protein. This implies that the near to the lipid-water interphase ends of transmembrane protein segments in contact with the lipid bilayer such as the M4, are likely candidates to be involved in determining the observed selectivity.

As the main conclusion of this report, we propose that a protein-induced, lipid phase separation phenomenon, causing the segregation of specific lipid species, constitutes the basis for the lipid modulation of AcChR structure and function. Partly supported by grant PM95-0108 from the DGICYT of Spain.

References

- Raines, D.E., Miller, K.W. (1993) The role of the charged lipid selectivity for the nicotinic acetylcholine receptor. *Biophys. J.* 64, 632-641
- Fong, T.M., McNamee, M.G. (1987) Stabilization of acetylcholine receptor secondary structure by cholesterol and negatively charged phospholipids in membranes. *Biochemistry* 26, 3871-3880
- Echabe, I., Encinar, J.A., Arrondo, J.L.R. (1997) Effect of noise on the obtention of protein structure by band decomposition of the infrared spectrum. *Biospectroscopy* 3, 469-475

35

4-Oxystilbene compounds are selective antagonists for neuronal nicotinic α -bungarotoxin receptors

C. Gotti^{a, b}, M. Moretti^{a, b}, B. Balestra^{a, b}, L. Maggi^d, F. Eusebi^c, E. Palma^c, G. E. Rovati^c, L. Villa^d, M. Pallavicini^d, F. Berti^b, G. Rossoni^b, F. Clementi^{a, b}

^aCNR Cell. Mol. Pharmacol. Ctr., ^bDept. Med. Pharmacol., ^cInst. Pharmacol. Sci., ^dInst. Pharmaceutical Chem. Toxicol., Univ. of Milan, ^eDept. Exp. Medicine, Univ. 'La Sapienza', Rome, Italy

As the pharmacological properties and physiological functions of nAChR subtypes are still largely unknown, great efforts have been made to investigate their structure in order to provide information that can be used to design more selective compounds. Much of the recent increase in nicotinic ligand research is due to the growing evidence that nAChRs are involved in brain function, and play a central role in the physiopathology of a number of disorders (1, 2)

It has long been known that 4-oxystilbene derivatives have ganglioplegic activity (3) and are able to antagonise nicotine-induced tremors in rabbits. We therefore decided to test the selectivity of the five different 4-oxystilbene compounds on nAChR subtypes. Binding experiments showed that the most active was the old drug MG624 that specifically inhibits the binding of ¹²⁵I- α Bgtx to the α 7-containing receptors and, although to a much lesser extent, the binding of ³H-Epi to the β 4 and β 2-containing receptors in a heterogeneous receptor population. In order to clarify the pharmacological profile of this compound, we studied its capacity to bind the individual neuronal subtypes, and compared the results with the effects obtained in the same subtypes expressed in oocytes. It was found that MG624 inhibits the binding of ¹²⁵I- α -bungarotoxin (α Bgtx) to the α 7 subtype and that of ³H-epibatidine (Epi) to the α 4 β 2 subtype with K_i values of respectively 106 nM and 84 μ M. The drug also inhibits ACh elicited currents (IACH)

on the oocyte-expressed α 7 subtype and the α 4 β 2 subtype at half-inhibitory concentrations (IC₅₀) of respectively 109 nM and 3.2 μ M.

In order to explore the interaction of MG624 with the α 7 subtype, we took advantage of the expression in *Xenopus* oocytes of an α 7 homomeric mutant receptor with a threonine-for-leucine 247 substitution (L247T α 7) (4). Whereas MG624 did not induce any current on oocytes expressing the wild type α 7 receptor, it did induce large currents on the oocyte-expressed L247T α 7 receptor. The MG624 elicited current (IMG624) on the L247T α 7 receptor has an EC₅₀ of 0.2 nM and a Hill coefficient nH of 1.9, and is blocked by the nicotinic receptor antagonist methyllycaconitine (0.5 nM). These electrophysiological studies show that MG624 is a potent antagonist of neuronal α 7 nAChR, which becomes a competitive agonist following the mutation of the highly conserved leucine residue 247 located in the M2 channel domain.

References

- 1) Léna C and J-P Changeux (1997) *Curr Op Neurobiol* 7:674-682
- 2) Lindstrom J (1997) *Mol Neurobiol* 15: 193-222
- 3) Mantegazza, P and Tommasini, R (1955) *Arch Int Pharmacodyn* 4:371-403
- 4) Bertrand D et al (1992) *Proc Natl Acad Sci USA* 89:1261-1265

36

Regulation of utrophin expression during development of skeletal muscle cells

A.O. Gramolini, J.A. Lunde, J. Wu, B.J. Jasmin

Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5

Utrophin is a large cytoskeletal protein of the spectrin superfamily that displays a high degree of sequence identity with dystrophin. In contrast to the latter which accumulates at the cytoplasmic face along the sarcolemma of normal muscle fibers, utrophin is preferentially expressed at the postsynaptic membrane of the neuromuscular junction in both normal and dystrophic muscle fibers. Since upregulation of utrophin levels into extrasynaptic compartments of muscle fibers is currently envisaged as a potential therapeutic strategy to counteract the effects of Duchenne muscular dystrophy (see 1 for example), it thus appears important to decipher the cellular and molecular mechanisms presiding over utrophin expression in muscle. We have recently begun a first series of studies aimed at identifying the regulatory events involved in maintaining the restricted expression of utrophin at the adult neuromuscular synapse. In these initial studies, we have shown that utrophin mRNAs accumulate within the postsynaptic sarcoplasm as a result of the local activation of the utrophin gene within the synaptic myonuclei (2). In addition, we demonstrated that the nerve exerts a profound influence on the local expression of utro-

phin through the release of nerve-derived trophic factors which culminate in the transactivation of the utrophin gene via an N-box motif (3). Aside from identifying the signal transduction machinery responsible for maintaining expression of the utrophin gene within synaptic myonuclei, it appears also crucial to determine the factors leading to the repression of its expression in extrasynaptic compartments of muscle fibers (see 4). In this context, we have previously shown that, in contrast to the expression of the mRNAs encoding acetylcholinesterase and the various subunits of the acetylcholine receptor (AChR), expression of utrophin is largely insensitive to abolition of nerve-evoked electrical activity (5). In attempts to gain insights into the mechanisms preventing expression of significant amounts of utrophin in extrasynaptic compartments of muscle fibers, we have, in the present study, examined the cellular and molecular mechanisms regulating utrophin expression during the early stages of myogenesis and synaptogenesis using distinct yet, complementary approaches.

First, we examined utrophin mRNA expression in developing muscles to determine the time point at which these transcripts

become enriched within the postsynaptic sarcoplasm. To this end, we thus performed *in situ* hybridization experiments with synthetic oligonucleotides specific for utrophin transcripts on skeletal muscles obtained from embryonic to neo-natal mice. Results of these studies showed that compartmentalization of utrophin transcripts within the junctional region of muscle fibers occurs at early stages of muscle development and synapse formation.

Next, we studied the pattern of expression of utrophin during differentiation of C2 muscle cells grown in culture. In these experiments, we determined that utrophin levels increase by approximately 2-fold during fusion of myoblasts into multinucleated myotubes. This increase in protein expression was accompanied by a parallel elevation in the levels of utrophin transcripts. Interestingly, this increase in utrophin expression was blocked by treating the myoblasts with EGTA which prevents fusion thereby indicating that the changes in utrophin expression during myogenic differentiation are linked to alterations in the cellular architecture which occur upon fusion and are independent of the activation of the myogenic differentiation program. To determine the contribution of transcriptional versus post-transcriptional regulatory mechanisms in the control of utrophin mRNA levels during differentiation, we measured the transcriptional activity of the utrophin gene by nuclear run-on assays and observed that a modest increase in the rate of transcription in nuclei isolated from differentiated myotubes. We also determined that the half-life for utrophin mRNAs in myoblasts was 20 h and that it remained essentially unchanged in myotubes. These data indicate therefore that the increased expression of utrophin in myotubes is transcriptionally regulated and does not involve alterations in the stability of utrophin transcripts. In addition, these studies show that in contrast to several other synaptic and cytoskeletal proteins including AChR, AChE and dystrophin, expression of utrophin is only modestly increased during differentiation of myogenic cells. In separate experiments, we also determined that utrophin mRNAs

preferentially associate with cytoskeleton-bound polysomes attached to the actin microfilament meshwork and that the extent of this association appears developmentally regulated since utrophin mRNAs are not found enriched within this polysomal fraction in myoblasts. These latter findings suggest that targeting of utrophin transcripts to the translational machinery is subject to developmental influences.

Finally, to determine whether the modest increase in utrophin mRNA expression seen during myogenic differentiation of cells grown in culture, also occurs *in vivo*, we injected soleus muscles with cardiotoxin which induced muscle fiber degeneration, and followed the pattern of expression of utrophin transcripts during the period of regeneration. In contrast to a large increase in dystrophin mRNA levels that occurred during regeneration, the abundance of utrophin transcripts remained relatively constant over this period as expected on the basis of the experiments described above. Since fetal and regenerating muscle fibers are known to contain high levels of utrophin, these data suggest therefore that post-translational regulatory mechanisms also play a predominant role in dictating utrophin levels in muscle. This work is supported by grants from the Association Française Contre les Myopathies, the Muscular Dystrophy Association of America and the Medical Research Council of Canada. A.O.G. is an Arthur Minden Predoctoral Fellow of the Muscular Dystrophy Association of Canada. B.J.J. is a Scholar of the Medical Research Council of Canada.

References

- (1) Tinsley et al., *Nature* 384: 349–353, 1996
- (2) Gramolini et al., *J. Biol. Chem.* 272: 8117–8120
- (3) Gramolini et al., *J. Biol. Chem.* 273: 736–743, 1998
- (4) Jasmin et al., *FEBS Lett.* 374: 393–398, 1995
- (5) Gramolini and Jasmin, *Neuromus. Dis.* in press, 1998.

37

3.5 kb AChE mRNA is more sensitive to degradation than 2.3 kb AChE mRNA in the denervated fast rat skeletal muscle

Z. Grubic, S. Kreft, K. Zajc-Kreft

Dpt. of Molecular Neurobiology, Institute of Pathophysiology, Medical School, University of Ljubljana, 1000 Ljubljana, Zaloška 4, Slovenia

Introduction

Acetylcholinesterase (EC. 3.1.1.7, AChE) is an essential functional component of the neuromuscular junction (reviewed by Hall and Sanes, 1993 and Massoulié et al., 1993). In the mammalian skeletal muscle, AChE mRNA is represented by two species (Rachinsky et al., 1990) originating from the alternate use of the two polyadenylation signals (Li et al., 1991). The 2.3 kb species encodes the hydrophilic form of AChE catalytic subunit; this size reflects the distance from the cap site to the first polyadenylation signal in the AChE gene. A second polyadenylation signal was found 1.2 kb in the 3' direction and its usage gives rise to the 3.5 kb species of AChE mRNA (Li et al., 1991). The role of divergency of 3' untranslated region of AChE mRNA is unknown. Additional sequence of the long transcript contains AU rich elements (Li et al., 1991; Fuentes and Taylor, 1993), which were found to influence the stability of several mRNAs (Greenberg and Belasco, 1993).

To elucidate the mechanisms underlying decreased AChE mRNA level in the denervated muscle we investigated in this study if the two AChE mRNA species differ in their sensitivity towards degradation under such conditions. We followed the time course of the AChE mRNA decrease after denervation of rat sternomastoideus muscle. We also studied degradation of both AChE mRNA species after their exposure to the subcellular fractions isolated from control and denervated muscles.

Experimental procedures

All experiments were carried out on the sternomastoideus muscles isolated from the female Wistar strain rats of about 190 g. AChE mRNA was determined by non-radioactive Northern blotting in the mechanically denervated muscle after selected time intervals. Digoxigenine labeled RNA, corresponding to the part of the second exon of the AChE gene (nt. 370–706) was used as a probe. In our *in vitro* model, total deproteinated RNA, isolated from the

Table I. Degradation of longer (3.5 kb) and shorter (2.3 kb) AChE mRNA species in the denervated rat sternomastoideus muscle. Each column represents means of densitometric analyses of two independent Northern blots. Results of densitometric analyses are expressed in relative units: control level of 2.3 kb AChE mRNA was taken as a reference (100 units).

AChE mRNA species	Control	2 days after denervation	5 days after denervation	8 days after denervation
3.5 kb AChE mRNA	96	15	13.5	0
2.3 kb AChE mRNA	100	20	34	20

sternomastoideus muscle, was exposed to subcellular muscular fractions prepared from another sternomastoideus muscle, which was either normal or denervated. Remaining AChE mRNA was determined after selected time intervals.

Results

As revealed by our Northern blot analyses, a longer AChE mRNA transcript (3.5 kb) practically disappears after 5–6 of denervation. On the other hand, the level of shorter species (2.3 kb) decreases during the first 2 days to about 20% of its control value, but then remains at this level in the subsequent six days (table I). A small portion of AChE activity (< 30% of control activity) persisting in the denervated muscle 8 days after denervation seems therefore to be synthesized exclusively from the 2.3 species. Degradation of AChE mRNA was not specific, since α -actin mRNA was also affected. 3.5 kb AChE mRNA was degraded faster also under the in vitro conditions, suggesting that its reduced level in the denervated muscle indeed reflects higher degradation rate and not just its decreased synthesis.

Discussion

Unlike myoblast fusion of the C2 cells, during which AChE mRNA increases at the unchanged proportion of both AChE mRNA species (Fuentes and Taylor, 1993), 8 days of denervation of the fast rat muscle practically eliminates 3.5 kb AChE mRNA,

but leaves a small portion of 2.3 kb species virtually intact. A functional meaning of expression of 2.3 kb species could therefore be to prevent complete blockade of AChE synthesis at the AChE mRNA level under the stressful conditions like denervation, which destabilizes several RNAs. On the other hand, expression of longer AChE transcript might serve to some yet unknown control of AChE synthesis in the skeletal muscle at the mRNA level.

References

- Fuentes ME and Taylor P (1993) Control of acetylcholinesterase gene expression during myogenesis. *Neuron* 10, 679–687
- Greenberg ME and Belasco JG (1993) Control of the decay of labile protooncogene and cytokine mRNAs. In: *Messenger RNA stability*, J Belasco and G Brawerman, eds. San Diego, New York, Academic Press, 199–218
- Hall ZW and Sanes JR (1993) Synaptic structure and development: The neuromuscular junction. *Cell* 72/Neuron 10 (Suppl.), 99–121
- Li Y, Camp S, Rachinsky TL, Getman D, Taylor P (1991) Gene structure of mammalian acetylcholinesterase. *J. Biol. Chem.* 260, 23083–23090
- Massoulié J, Pezzementi L, Bon S, Krejci E, Valette FM (1993) Molecular and cellular biology of cholinesterases. *Prog. Neurobiol.* 41, 31–91
- Rachinsky TL, Camp S, Li Y, Ekstrom TJ, Newton M and Taylor P (1990) Molecular cloning of mouse acetylcholinesterase: tissue distribution of alternatively spliced mRNA species. *Neuron* 5, 317–327

38 Photoaffinity labeling of nicotinic acetylcholine receptor with a tritiated agonist: A new tool for investigating the functional, activated state

T. Grutter, M. Goeldner, F. Kotzyba-Hibert

Laboratoire de chimie bioorganique, UMR 7514 CNRS, Faculté de Pharmacie, Université Louis Pasteur, Strasbourg, BP 24, 67401 Illkirch cedex, France

Upon agonist activation, the nicotinic acetylcholine receptor (AChR) undergoes allosteric transitions leading to channel opening and sodium ion influx. The molecular structure of *Torpedo marmorata* acetylcholine binding site has been investigated previously by photoaffinity labeling and delineated by three different loops on the α -subunit (loop A, Trp-86 and Tyr-93; loop B, Trp-149 and Tyr-151; and loop C, Tyr-190, Cys-192, Cys-193, and Tyr-198). However, most of the photosensitive probes used for this purpose interacted only with closed receptor states (1, 2). As the transition from the resting (R) to the active state (A) takes place on a millisecond time scale, photosensitive agonists which generate highly reactive species are required in order to label the functional state (A) of AChR (figure 1).

Thus, we have synthesized and developed a new photoactive agonist (PA) of AChR with functional activity on human TE 671 cell lines (3). In the absence of light, PA has micromolar affinity for ACh binding site on the D state (after proadifen preincubation) with a 10-fold decrease in affinity for the native form (without proadifen) as expected for cholinergic ligands. [3 H]PA was synthesized and used for photolabeling experiments (figure 2). Upon UV irradiation (360 nm), [3 H]PA labels covalently AChR-rich membranes whereas less than 0.5% labeling occurs in the absence of light. The pattern of [3 H]PA labeling on the different subunits of the AChR in the D state shows a predominant incorporation into the α -subunit with minor incorporation into the γ -subunit, both in a protectable manner (~ 70 to 80% protection)

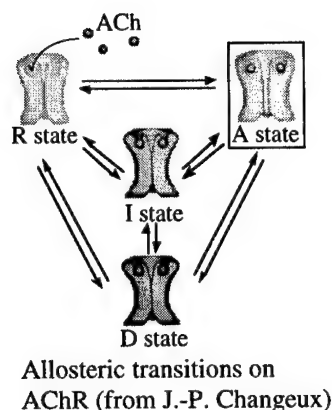


Figure 1. Allosteric transitions on AChR (from J.-P. Changeux).

with either agonist (carbamylcholine) or antagonists (d-tubocurarine, hexamethonium and α -bungarotoxin). The stoichiometry of photocoupling was measured: 1 mol of [3 H]PA is incorporated per mol of α -bungarotoxin binding site inactivated demonstrating the high efficiency of [3 H]PA as photoalkylating agent. The specific incorporation of [3 H]PA into the α and γ subunits is saturable for increasing probe concentration. Preparative photolabeling yields about 35 pmol of site-specific alkylated material per experiment ($\sim 7\%$ of the total amount of ACh binding site into α -subunit) allowing us to prepare large scale of photolabeled AChR.

In a first step, large amounts of [3 H]PA alkylated polypeptides will lead us, after proteolytic cleavages, purification of tritiated peptides and microsequencing, to characterize the amino acids belonging to ACh binding sites in the D state.

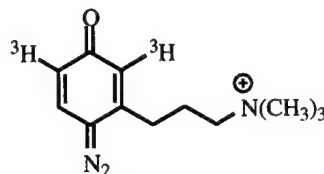


Figure 2. Structure of the photoactivatable agonist [3 H]PA.

In a second step, we plan to do time-resolved photolabeling with rapid mixing apparatus combined with laser photoirradiation. These experiments might permit us to label AChR with [3 H]PA on its functional activated state A.

A comparative study at the molecular level of the dynamic photolabeling experiments and those obtained at equilibrium for the D state may elucidate the topographical changes occurring at the ACh binding sites during agonist activation.

References

- 1 Dennis, M., Giraudat, J., Kotzyba-Hibert, F., Goeldner, M., Hirth, C., Chang, J.-Y., Lazure, C., Chrétien, M., and Changeux, J.-P. (1988) *Biochemistry* 27, 2337-2345
- 2 Galzi, J.-L., Black, D., Goeldner, M., Hirth, C., and Changeux, J.-P. (1990) *J. Biol. Chem.* 265, 10430-10437
- 3 Kotzyba-Hibert, F., Kessler, P., Zeribib, V., Grutter T., Bogen, C., Takeda, K., Hammadi, A., Knerr, L., and Goeldner, M. (1997) *Bioconj. Chem.* 8, 472-480

Muscarinic receptors (M2, M3) in rat and guinea pig thoracic dorsal root ganglia

R. Haberberger, M. Henrich, W. Kummer

Institut für Anatomie und Zellbiologie, Justus-Liebig-Universität, Aulweg 123, 35385 Giessen, Germany

Acetylcholine (ACh) excites mechanoreceptors and produces pain when applied to human skin. In the rat skin, a group of carbachol sensitive C-nociceptors can be excited by muscarine (7). Binding sites for ACh are present in rat dorsal root ganglia (DRG) and accumulate at the proximal site of a sciatic nerve ligature (8). Functional studies of cultured neurons from rat DRG show that stimulation of muscarinic receptors is followed by activation of the nitric oxide (NO)-cGMP signalling system (2), and muscarinic regulation of Ca^{2+} -currents has also been observed (9). For a better understanding of cholinergic regulation of sensory neuron function, the localization and distribution of muscarinic receptors, in particular its localization to defined subpopulations of sensory neurons, is of interest. Therefore, in the present study, thoracic DRG of rat and guinea pig were examined using histochemical and immunohistochemical methods for the presence of muscarinic M2- and M3-receptors (M2R, M3R) and markers for select neuronal populations. Sensory neurons of DRG can be subdivided in different groups including capsaicin-sensitive, non-peptidergic, small sensory neurons with binding sites for the α -D-galactose specific lectin, IB4 (6), and small-sized primary sensory neurons

containing the peptide, substance P (SP, 5). A group of SP-containing neurons also shows positive NADPH-diaphorase reaction (1) which indicates the presence of nitric oxide synthase (NOS, 3). Thus, IB4, NOS/NADPH-diaphorase reaction, and SP were used as markers for further characterization of M2R- and M3R-immunoreactive (-IR) sensory neurons. These investigations were performed at formaldehyde-fixed tissues processed either as cryostat sections or as free floating sections of polyethyleneglycol-embedded specimens.

Both rat and guinea pig DRG contained neurons with immunoreactivity for both subtypes of muscarinic receptors; the labeling densities of individual neurons varied over a wide range. In guinea pig thoracic DRG, M2R- and M3R-IR were present in nerve cell somata, axonal processes and in the endothelium and smooth muscle cells of intraganglionic blood vessels. Intense filamentous M3R-IR was observed in perikarya of medium-sized neurons (25–30 μ m in diameter) which mostly expressed IB4 binding sites. These cells contained neither NADPH-diaphorase activity nor SP-IR. Intense to slight M3R-IR was present in larger somata (40–50 μ m in diameter) which exhibit IB-4 binding sites

and, occasionally, SP-IR and/or NADPH-diaphorase activity. M2R-IR was predominantly present in larger neurons, both with and without I-B4 binding sites. A minor population of medium-sized neurons contained also M2R-IR and I-B4 binding sites. None of the M2R-IR neurons expressed SP-IR. In rat thoracic DRG, M2R- and M3R-IR were present in medium-sized and larger neurons. Predominantly, they expressed I-B4 binding sites, but some perikarya without IB-4 staining were also seen. Medium-sized M2R-IR perikarya often showed SP-IR and positive NADPH-diaphorase reaction whereas medium-sized M2R-IR perikarya and all M3R-IR neurons were virtually devoid of SP-IR and NADPH-diaphorase activity.

The present demonstration of M2R-IR on SP-IR neurons is in accordance with previous pharmacological and electrophysiological experiments describing M2R or M4R dependent inhibition of peptide release from sensory nerve terminals (4, 9). On the other hand, perikarya with intense M3R-IR contained neither SP-IR nor NADPH-diaphorase activity, but expressed I-B4 binding sites. Thus, presumably capsaicin-sensitive, non-peptidergic nociceptive neurons bear M3R. Accordingly, the ACh-dependent NO/cGMP signalling pathway can be abolished by capsaicin treatment (2). This study was supported by SFB 547, project C2

References

- 1) Aimi, Y., Fujimura, M., Vincent, S. R., Kimura, H. Localization of NADPH-diaphorase-containing neurons in sensory ganglia of the rat, *J. Comp. Neurol.*, 306 (1991) 382–392.
- 2) Bauer, M. B., Murphy, S., Gebhart, G. F. Muscarinic cholinergic stimulation of the nitric oxide-cyclic GMP signaling system in cultured rat sensory neurons, *Neurosci.*, 62 (1994) 351–359.
- 3) Dawson, T. D., Bredt, D. S., Fotuhi, M., Hwang, M., Snyder S. H. Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues, *Proc. Natl. Acad. Sci. USA* 88 (1991) 7797–7801.
- 4) Delaunois, A., Gustin, P., Ansay, M. Multiple muscarinic receptor subtypes mediating pulmonary oedema in the rabbit, *Pulm. Pharmacol.*, 7 (1994) 185–193.
- 5) Hökfelt, T., Kellerth, J. O., Nilsson, G., Pernow, B. Substance P: localization in central nervous system and in some primary sensory neurons, *Science* 190 (1975) 889–890.
- 6) Plenderleith, M. B., Snow, P. J. The plant lectin *Bandeiraea simplicifolia* I-B4 identifies a subpopulation of small diameter primary sensory neurones which innervate the skin in the rat, *Neurosci. Lett.*, 159 (1993) 17–20.
- 7) Steen, K. H., Reeh, P. W. Actions of cholinergic agonists and antagonists on sensory nerve endings in rat skin, *in vitro*, *J. Neurophysiol.*, 70 (1993) 397–405.
- 8) Wamsley, J. K., Zarbin, M. A., Kuhar, M. J. Muscarinic cholinergic receptors flow in the sciatic nerve, *Brain Res.*, 217 (1981) 155–161.
- 9) Wanke, E., Bianchi, L., Mantegazza, M., Guatteo, E., Macinelli, E., Ferroni, A. Muscarinic regulation of Ca^{2+} currents in rat sensory neurons: channel and receptor types, dose-response relationships and cross-talk pathways, *Eur. J. Neurosci.*, 6 (1994) 381–391.

40

Expression of nicotinic receptor α and β subunits in the prenatal and aged human brain

E. Hellström-Lindahl^a, O. Gorbounova^a, Å. Seiger^b, M. Mousavi^a, A. Nordberg^a

Department of Clinical Neuroscience and Family Medicine, ^aDivision of Molecular Neuropharmacology, ^bDivision of Geriatric Medicine, Karolinska institutet, Huddinge University Hospital, B84, S-141 86 Huddinge, Sweden

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels comprised of α and β subunits. Presently six α ($\alpha 2$ – $\alpha 7$) and three β ($\beta 2$ – $\beta 4$) subunits have been identified and cloned from human brain (Mc Gehee and Role, 1995; Elliott et al, 1996; Gotti et al, 1997). Precise subunit composition of the various subtypes and their regional distribution in human brain is still unclear. The nAChRs appear to have a functional role during brain development, since periods of transient high receptor density have been reported in the frontal cortex, hippocampus, cerebellum and brain stem in humans during mid-gestation and neonatal periods (Kinney et al, 1993; Court and Clementi, 1995). The earliest reported binding of [³H]-nicotine so far has been demonstrated in whole brain homogenates from 12-week-old fetuses (Cairns and Wonnacott, 1988) and choline acetyltransferase and acetylcholine esterase activity has been detected in human forebrain from 8–9 gestational weeks (Candy et al, 1985; Perry et al, 1986). Few data are available concerning the ontogenesis of nAChRs in brain during the first trimester. Moreover, there are no reports on their regional distribution and the gene expression of the different nAChR subunits. In this study the development of nicotinic acetylcholine receptors (nAChRs) in brains from human fetuses of 4–12 weeks gestational age was investigated. By using RT-PCR technique the expression of $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$, $\beta 3$ and $\beta 4$ mRNA subunits were all detected in the pre-

natal spinal cord, medulla oblongata, pons, cerebellum, mesencephalon, subcortical forebrain and cerebral cortex during first trimester development. Relative quantification of mRNA (Hellström-Lindahl et al., 1997) showed that highest levels for $\alpha 3$, $\alpha 4$ and $\alpha 7$ were expressed in spinal cord and $\alpha 5$ was most abundant in cortex. For the β -subunit mRNAs, $\beta 2$ was high in cortex and cerebellum, $\beta 3$ was highest in cerebellum whereas $\beta 4$ seemed to be equally distributed in all regions. A comparison of expression of nAChR subunit mRNAs in the cortex and cerebellum of prenatal and aged (54–81 years) brain showed that mRNA levels for $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ were all significantly higher in prenatal cortex and cerebellum than in aged brain, whereas the level of $\alpha 3$ transcript was similar, and $\beta 3$ significantly higher in aged cortex.

Specific binding of [³H]-epibatidine to prenatal brain membranes was detected as early as 4–5 weeks of gestation in many brain regions and the number of nAChRs increased with gestational age. The highest specific binding of [³H]-epibatidine and [³H]-cytisine was detected in spinal cord, pons and medulla oblongata and the lowest in cortex. Saturation analysis of binding data for both prenatal and aged brain revealed a single site for [³H]-cytisine and two binding sites for [³H]-epibatidine. The B_{max} values obtained with [³H]-cytisine and [³H]-epibatidine were about five and ten times higher for prenatal whole brain compared to aged frontal cortex. The reduced levels of nAChR subunit

mRNAs in aged brain shown in the present study is consistent with the earlier reported loss of nAChRs in cerebral cortex and hippocampus during normal aging (Nordberg et al., 1992; Court and Clementi, 1995). The early presence of nAChR proteins and gene transcripts observed suggests an important role for nAChRs in modulating dendritic outgrowth, establishment of neuronal connections and synaptogenesis during development.

References

- Cairns NJ, Wonnacott S (1988) [3 H] (-)-nicotine binding sites in fetal human brain. *Brain Res* 475, 1–7
- Candy JM, Perry EK, Perry RH, Bloxham CA, Thompson J, Johnson M, Oakley EA, Edwardson JA (1985) Evidence for the early pre-natal development of cortical cholinergic afferents from the nucleus of Meynert in the human foetus. *Neurosci Lett* 61, 91–95
- Court J, Clementi F (1995) Distribution of nicotinic subtypes in human brain. *Alzheimer Dis Assoc Disorders* 9 (Suppl. 2), 6–14
- Elliott KJ, Ellis SB, Berckhan KJ, Urrutia A, Chavez-Noriega LE, Johnson EC, Velicelebi G, Harpold MM (1996) Comparative structure of human neuronal $\alpha 2$ - $\alpha 7$ and $\beta 2$ - $\beta 4$ nicotinic acetylcholine receptor subunits and functional expression of the $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 7$, $\beta 2$ and $\beta 4$ subunits. *J Mol Neurosci* 7, 217–228
- Gotti C, Fornasari D, Clementi F (1997) Human neuronal nicotinic receptors. *Progr. Neurobiol* 53, 199–237
- Hellström-Lindahl E, Zhang X, Nordberg A (1997) Expression of nicotinic receptor subunit mRNAs in lymphocytes from normal and patients with Alzheimer's disease. *Alzheimer's Res* 3, 29–36
- Kinney HC, O'Donell TJ, Kriger P, Frost White PW (1993) Early developmental changes in [3 H] nicotine binding in the human brainstem. *Neurosci* 55, 1127–1138
- Nordberg A, Alafuzoff I, Winblad B (1992) Nicotinic and muscarinic subtypes in the human brain: Changes with aging and dementia. *J Neurosci Res* 31, 103–111

41

Stimulatory influence of ethanol on the septohippocampal cholinergic pathway. A role for GABA receptors?

C. Henn, J. Klein, K. Löffelholz

Dept. of Pharmacology, University of Mainz, Obere Zahlbacher Str. 67, D-55101 Mainz, Germany

The cholinergic septohippocampal pathway is involved in the processes of learning and memory and is a possible target for the cognitive dysfunction and amnesia observed after acute ethanol intake. Decreases of cortical cholinergic functions after high doses of ethanol have already been described using the push-pull technique (see ref. 2), measurements of ACh tissue levels (see ref. 2) and ACh release from brain slices (see ref. 4). An impairment of the hippocampal cholinergic system has so far only been observed after long-term ethanol administration (see ref. 1).

In the present study we used the microdialysis technique in combination with a sensitive HPLC system to monitor simultaneously changes of rat hippocampal acetylcholine (ACh) release and hippocampal ethanol levels upon acute ethanol administration. For this purpose, ethanol was administered either systemically (i.p.) or locally (via the probe). Samples were collected in 15-min intervals for estimation of acetylcholine and ethanol levels, respectively. Details of the microdialysis procedure and ACh determination were as described previously (see ref. 5).

In the present microdialysis study we report, to our knowledge for the first time, a stimulatory effect of ethanol upon the central cholinergic system in vivo. This was observed after systemic administration of low doses of ethanol as well as during local infusions into the hippocampus or the basal forebrain. The i.p. injection of low doses of ethanol (0.8 g/kg) led to a delayed stimulation of basal ACh release which occurred only 2 h past injection, i.e., at a time when hippocampal ethanol levels had already dropped to concentrations below 5 mM. The effect lasted for approximately 2 h (see ref. 3).

Similarly local infusion of ethanol via retrograde dialysis into the hippocampus (50 and 100 mM, respectively) led to a concentration-dependent increase of basal ACh release after 30 min of ethanol infusion. ACh levels remained elevated for up to 1 h after cessation of ethanol infusion when hippocampal ethanol levels had already dropped to zero.

In order to test whether GABAergic mechanisms may be involved in ethanol's actions, we used vigabatrin (GVG), an irre-

versible inhibitor of GABA transaminase. Vigabatrin, administered systemically (0.8 g/kg and 2.4 g/kg, respectively), led to a dose-dependent stimulation of hippocampal ACh release with maximum ACh levels occurring after 15 and 75 min, respectively ($159 \pm 9\%$ and $173 \pm 14\%$ of basal efflux, respectively; $n = 4$; see figure 1). Local infusions (1 and 10 μ M, respectively) also caused a concentration-dependent stimulation of hippocampal ACh release, but only after termination of vigabatrin-infusion. ACh levels returned to basal levels within 1 h.

The present study describes stimulatory effects of ethanol in low doses upon hippocampal ACh release which, seem to be due to a local effect of ethanol on the cholinergic septohippocampal pathway. The present data also indicate that these effects may be mediated by an involvement of a GABAergic mechanism, as both, ethanol and vigabatrin, lead to a stimulation of the hippocampal ACh outflow, possibly via stimulation of a disinhibitory GABA circuit.

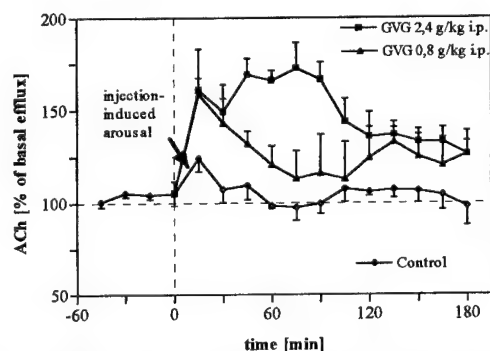


Figure 1. Effects of i.p. administration at $t = 0$ of 0.8 g/kg and 2.4 g/kg vigabatrin, respectively, on the basal outflow of ACh from the ventral rat hippocampus. ACh output is expressed as percent of basal efflux (1.57 ± 0.21 pmol/15 min); each data point represents the mean \pm S.E.M. of four experiments. Open symbols represent controls (1.5 mL saline i.p.); filled triangles 0.8 g/kg vigabatrin and filled squares 2.4 g/kg vigabatrin.

References

- (1) Casamenti F, Scali C, Vannucchi MG, Bartolini L and Pepeu G (1993): Long-term ethanol consumption by rats: effect on acetylcholine release in vivo, choline acetyltransferase activity, and behaviour. *Neuroscience* 56, 465-471.
- (2) Erickson CK, Graham DT (1973): Alteration of cortical and reticular acetylcholine release by ethanol in vivo. *J Pharmacol Exp Ther* 185: 583 - 93
- (3) Henn C, Löffelholz K, Klein J (1998): Stimulatory and inhibitory effects of ethanol on hippocampal acetylcholine release. *Naunyn Schmiedeberg's Arch Pharmacol* (in press)
- (4) Kalant H, Grose W (1967): Effects of ethanol and pentobarbital on release of acetylcholine from cerebral cortex slices. *J Pharmacol Exp Ther* 158: 386 - 93.
- (5) Köppen A, Klein J, Schmidt BH, van der Staay F-J and Löffelholz K (1996): Effects of nicotinamide on central cholinergic transmission and on spatial learning in rats. *Pharmacol. Biochem. Behav.* 53, 783-790.

42

ADP-ribosyl cyclase coupled with muscarinic acetylcholine receptors via G proteins in NG108-15 cells

H. Higashida

Department of Biophysical Genetics, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan

Cyclic adenosine diphosphate ribose (cADPR) is synthesized from β -NAD⁺ by ADP-ribosyl cyclase. Pharmacological studies suggest that cADPR is an endogenous modulator of Ca²⁺-induced Ca²⁺ release from ryanodine-sensitive Ca²⁺ stores. However, the question whether or not cADPR mediates the intracellular action of conventional hormones and neurotransmitters in mammalian cells remains unsolved. For cADPR to be accepted as an intracellular second messenger downstream of such receptors; in addition to its pharmacological effects, extracellular stimulation should activate (or inhibit) ADP-ribosyl cyclase. The typical pathway in mammalian cells consists of signals from receptors involved in forming intracellular second messengers, such as cyclic AMP or inositol-1,4,5-trisphosphate and diacylglycerol, being transduced to effector enzymes of adenylate cyclase or phospholipase C via G proteins in the cell surface membrane. Therefore, I examined whether or not cADPR formation is regulated by ADP-ribosyl cyclase through the direct action of G proteins in the cell membrane.

Stimulation of muscarinic acetylcholine receptors (mAChRs) by carbamylcholine (CCh) changed the rate of cADPR formation in NG108-15 neuroblastoma × glioma hybrid cells, in which the enzyme activity was found in crude membrane fractions. Interestingly, activation of ADP-ribosyl cyclase by CCh was observed

in membranes obtained from NG108-15 cells overexpressing m1 or m3 mAChRs, while inhibition was mediated by endogenous m4 mAChRs and exogenous m2 mAChRs (Higashida et al., 1997). These effects were mimicked by GTP in NG108-15 cells. The CCh-induced activation was inhibited by prior treatment of cells with cholera toxin, while the inhibition of ADP-ribosyl cyclase was sensitive to pertussis toxin. Although further validation is necessary to determine exactly which G proteins are involved in the stimulatory and inhibitory pathways to ADP-ribosyl cyclase, it can be hypothesized from these observations that the signal to ADP-ribosyl cyclase from mAChRs is mediated via G proteins (Higashida, 1997).

References

- Higashida H (1997) ADP-ribosyl cyclase coupled with receptors via G proteins. *FEBS Lett.* 418, 355-356
- Higashida H, Yokoyama S, Hashii M, Taketo M, Higashida M, Takayasu T, Ohshima T, Takasawa S, Okamoto H, Noda M (1997) Muscarinic receptor-mediated dual regulation of ADP-ribosyl cyclase in NG108-15 neuronal cell membranes. *J. Biol. Chem.* 272, 31272-31277

43

Muscarinic acetylcholine m1-m4 receptors overexpressed in neuroblastoma-glioma hybrid NG108-15 cells as a cholinergic model

H. Higashida, S. Yokoyama, N. Hoshi, M. Hashii, A. Egorova, Z.-G. Zhong

Department of Biophysical Genetics, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan

Neuroblastoma × glioma hybrid NG108-15 cells possess endogenous m4 muscarinic acetylcholine receptors (mAChRs), which do not couple to phospholipase C but couple to adenylate cyclase and voltage-dependent Ca²⁺ channels (Higashida et al., 1990). Four genetic subtypes of mAChRs differ in the effects when stimulated by muscarinic agonists in NG108-15 cells overexpressing each mAChR (Noda et al., 1996). Broadly speaking, the principle falls into two categories: the odd-numbered receptors (m1 and m3) activate phospholipase C, increase cellular inositol trisphosphate/Ca²⁺ levels. Furthermore, recently, we found that formation of cyclic ADP-ribose (cADPR) is increased

in a reaction mixture with crude membrane fractions obtained from NG108-15 cells overexpressing m1/m3 mAChRs by muscarinic stimulation (Higashida et al., 1997). This is associated with a transient decrease of the cellular NAD⁺ level in an intact cell. In contrast, the stimulation of even-numbered receptors (m2 and m4) result in an inhibition of ADP-ribosyl cyclase as well as adenylate cyclase and an decrease of N-type Ca²⁺ currents. Signals from m2/m4 mAChRs are discriminated by their sensitivity for pertussis toxin, while those from m1/m3 mAChRs are not. Thus, coupling to adenylate cyclase, phospholipase C, ADP-ribosyl cyclase and Ca²⁺ ion channels from mAChRs shows a

dichotomy in NG108-15 cells, which may reflect mAChR-induced responses in intact neurons in vivo (Higashida, 1997).

References

- Higashida H (1997) ADP-ribosyl cyclase coupled with receptors via G proteins. *FEBS Lett.* 418, 355–356.
- Higashida H, Hashii M, Fukuda K, Caulfield M P, Numa S & Brown D A (1990) Selective coupling of different muscarinic acetylcholine receptors to neuronal calcium current in DNA-transfected cells. *Proc. Roy. Soc. B* 242, 68–74.
- Higashida H, Yokoyama S, Hashii M, Taketo M, Higashida M, Takayasu T, Ohshima T, Takasawa S, Okamoto H, Noda M (1997) Muscarinic receptor-mediated dual regulation of ADP-ribosyl cyclase in NG108-15 neuronal cell membranes. *J. Biol. Chem.* 272, 31272–31277.
- Noda M, Ishizaka N, Yokoyama S, Hoshi N, Kimura Y, Hashii M, Taketo M, Egorova A, Knijnik R, Fukuda K, Morikawa H, Brown DA, Higashida H (1996) Inositol trisphosphate/Ca²⁺ as messengers of bradykinin B2 and muscarinic acetylcholine m1-m4 receptors in neuroblastoma-derived hybrid cells. *J. Lipid Mediators Cell Signal.* 14, 175–185.

44

Acute and delayed effects of nitrogen oxide on metabolism of acetyl moiety of acetylcholine in differentiated and non-differentiated hybrid SN56 neurons.

A. Jankowska^a, M. Tomaszewicz^a, B. Madziar^a, J.K. Blusztajn^b, A. Szutowicz^a

^aDepartment of Clinical Biochemistry, Medical University of Gdansk, Debinki 7, 80-211 Gdansk, Poland

^bDepartment of Pathology, Boston University School of Medicine, Boston, MA, USA

Loss of septal and cortical cholinergic neurons followed by development of mental deficits is a key feature of various degenerative brain diseases. This preferential vulnerability of brain cholinergic neurons to different pathogenic factors may be due to the fact that, in addition to energy production and structural lipid synthesis taking place in all types of neurons, they utilize acetyl-CoA for acetylcholine (ACh) synthesis (Szutowicz et al., 1996). Several pathologic conditions including hypoxia, excessive release of NO and glutamate that impair energy production and increase ACh release may contribute to this injury (Iadecola, 1997). On the other hand, various groups of cholinergic neurons were found to display a differential sensitivity to similar pathogenic inputs (Cooper and Sofroniew, 1996). The source of these differences is not known. Our studies have shown that in various brain regions, ratios of acetyl-CoA producing enzymes and choline acetyltransferase (ChAT) activities were significantly different (Szutowicz et al., 1982). Therefore, the aim of this work was to investigate if induction of cholinergic phenotype in SN56 septal hybrid cholinergic neurons may modify susceptibility of their acetyl-CoA and ACh metabolism to increased NO levels.

SN56 cells (26–32 passages) were plated ($2.5\text{--}2.7 \pm 10^6$ cells/30 cm²) and grown to confluency for 3 days in Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum at 37 °C in an atmosphere of 95% air and 5% CO₂. In control conditions cells maintained stable levels of expression of pyruvate dehydrogenase (PDH) and ChAT activities, equal to 7.7 ± 0.2 and 0.20 ± 0.007 nmol/min/mg protein, respectively. Addition of 1 mM dibutyl cAMP (dbcAMP) inhibited PDH activity for 29% but increased that of ChAT 110%. On the other hand, 0.001 mM all-trans-retinoic acid (RA) did not affect PDH and simultaneously raised activity of ChAT 145% (figure 1). Added together dbcAMP and RA suppressed PDH (24%) but exerted an additive activatory effect on ChAT activity (305%) (figure 1). Protein content in control was equal to 2.95 ± 0.28 per plate. It was reduced in RA, dbcAMP and dbcAMP + RA treated neurons to 2.57 ± 0.36 , 2.12 ± 0.25 and 1.39 ± 0.24 mg per plate, respectively. Drop of protein content in particular experimental group correlated with decreased cell density and increased neurite outgrowth.

Exposition of cells cultured for 2 days, to 1 mM sodium nitroprusside (SNP) for 10 min caused within the following 2 days

a decrease of PDH activity in all groups (24–35%) (figure 1A). Such treatment did not affect expression of ChAT activity in control and dbcAMP-treated cells (figure 1). On the other hand, it suppressed by 35% activity of ChAT in neurons differentiated with RA or with dbcAMP + RA (figure 1B). SNP did not affect protein content in control cells but decreased it in all experimental groups from 34 to 52%, without apparent changes in cell morphology (not shown).

Control and dbcAMP + RA-treated SN56 neurons when incubated in depolarizing medium (30 mM KCl), maintained stable level acetyl-CoA and steady rates of pyruvate utilization and ACh release, during the entire period of 30 min incubation at 37 °C. Addition of dbcAMP + RA to growth medium caused 35% inhibition of pyruvate utilization, 45% suppression of acetyl-CoA content and reduction of spontaneous and Ca-evoked ACh release for about 70% (figure 2). In control neurons 0.2 mM SNP caused 35% inhibition of pyruvate utilization and slight activation of ACh release at unchanged level of acetyl-CoA. In dbcAMP + RA treat-

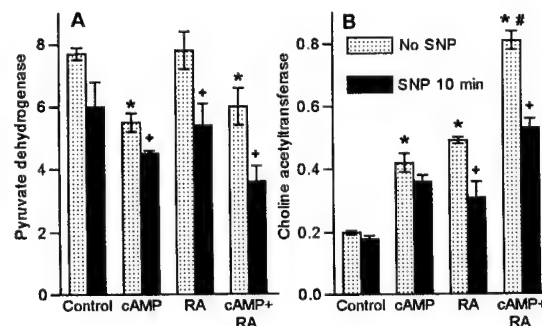


Figure 1. Effect of 10 min treatment with 1 mM SNP on posttreatment expression of PDH (A) and ChAT (B) activities (nmol/min/mg of protein) in SN56 cells non-differentiated (Control) and differentiated with db-cAMP (cAMP) and retinoic acid (RA). Results are means \pm S.E.M. of 5–10 experiments. $P < 0.001$ compared with respective control for ChAT and $P < 0.05$ for PDH; $^*P < 0.001$ compared with respective no SNP for ChAT and $P < 0.05$ for PDH; $^*P < 0.001$ compared with respective cAMP or RA conditions.

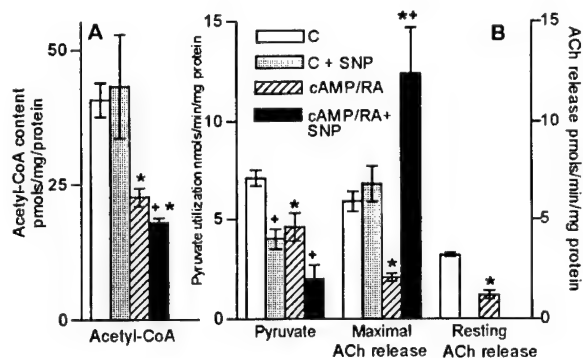


Figure 2. Effect of 0.2 mM SNP on (A) acetyl-CoA content, (B) pyruvate utilization, resting ACh release (no Ca in the medium) and maximal ACh release (1 mM Ca) in SN56 cells grown in basal conditions (C) or in the presence of 1 mM dbcAMP and 0.001 mM RA (cAMP/RA). Results are means \pm S.E.M. of 4–5 duplicate experiments. * P < 0.01 compared with respective control; ** P < 0.05 compared with respective no SNP conditions.

ted neurons, SNP brought about 50% inhibition of pyruvate utilization. However, unlike in controls, it caused a marked, six-fold increase of ACh release and 21% decrease of acetyl-CoA content (figure 2).

It is known that cholinergic differentiation of SN56 cells may proceed through the activation adenylate cyclase and directly through RA-activated transcription factors (Pedersen et al., 1995). Additive actions of dbcAMP and RA on ChAT activity indicate that expression of the cholinergic phenotype was mediated independently by these two intracellular signal transduction systems. It is possible that in vivo combined influences of multiple signaling pathways may provide highly variable levels of expression of cholinergic phenotype in particular groups of brain cholinergic neurons (Tomaszewicz et al., 1998). Decreased expression of PDH activity in differentiating neurons supports the hypothesis that rate of ACh synthesis is not tightly linked with the rate of acetyl-CoA generation in mitochondria (Szutowicz et al., 1996). The presumable efficacy of acetyl-CoA transport from mitochondria to cy-

toplasm is a rate-limiting factor for transmitter synthesis. However, in dbcAMP + RA treated cells rates of spontaneous and Ca-evoked ACh release were lower than in controls despite higher intracellular transmitter content. The source of this discrepancy remains to be found. One may conclude that it was not due to the low content of acetyl-CoA since activation of differentiated cells by SNP increased rates of ACh release/synthesis to much higher values than in non-differentiated ones. It might create increased demand for cytoplasmic acetyl-CoA and its shortage for mitochondria energy production as it was demonstrated here by the decrease of acetyl-CoA content in differentiated cells incubated with SNP (figure 2). Also, a short time of exposure of differentiating SN56 to SNP may impair by such mechanism expression of PDH, resulting in development of cholinergic neurons more susceptible to various degenerative insults (figure 2). These data may, at least in part, explain preferential susceptibility of septal cholinergic neurons to various neurotoxic inputs. This work was supported by K.B.N. project No 6P04A 013 10.

References

- Cooper JD, Sofroniew MV (1996) Increased vulnerability of septal cholinergic neurons to partial loss of target neurons in aged rats. *Neuroscience* 75, 29–35.
- Iadecola C (1997) Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci.* 20, 132–139.
- Pedersen WA, Berse B, Sch (ler U, Wainer BH, Blusztajn JK (1995) All-trans- and 9-cis-retinoic acid enhance the cholinergic properties of a murine septal cell line: Evidence that effects are mediated by activation of retinoic acid receptor- α . *J. Neurochem.* 65, 50–58.
- Szutowicz A, Tomaszewicz M, Bielarczyk H (1996) Disturbances of acetyl-CoA, energy and acetylcholine metabolism in some encephalopathies. *Acta Neurobiol. Exp.* 56, 323–339.
- Szutowicz A, Stepień M, Bielarczyk H, Kabata J, Lysiak W (1982) ATP-citrate lyase in cholinergic nerve endings. *Neurochem. Res.* 7, 798–810.
- Tomaszewicz M, Jankowska A, Madziar B, Blusztajn JK, Szutowicz A (1998) Effect of nerve growth factor and other differentiating agents on expression of enzymes of acetyl-CoA and acetylcholine metabolism in SN56 cholinergic cells. *J. Physiol. (Paris)* 92, in press

45 Molecular mechanisms underlying the synapse-specific expression and activity-linked regulation of acetylcholinesterase at the neuromuscular junction: Role of intronic sequences

B.J. Jasmin, R.Y.Y. Chan, C. Boudreau-Larivière, L. Angus, F.A. Mankal, A.M. Krupa

Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5

We have previously shown that mRNAs encoding acetylcholinesterase (AChE) are highly concentrated within the postsynaptic sarcoplasm of adult muscle fibers (1, 2) where their expression is markedly influenced by nerve-evoked electrical activity (2, 3). In a first series of experiments, we have therefore examined the relative contribution of transcriptional versus post-transcriptional regulatory mechanisms in the synaptic accumulation of AChE transcripts in skeletal muscle fibers. To this end, we have cloned a 4.7 kb DNA fragment upstream of the translation start site in the rat AChE gene and generated multiple promoter-reporter gene constructs containing LacZ and a nuclear localization signal (nlsLacZ). These constructs were directly injected into tibialis an-

terior muscles of mice and 14 days later, muscles were excised and frozen. Muscles were subsequently cut in a cryostat and tissue sections were histochemically stained for the demonstration of β -galactosidase expression. The position of blue myonuclei indicative of promoter activity, was compared to that of neuromuscular junctions identified by AChE histochemistry. Injections of promoter-reporter gene constructs containing DNA fragments ranging from 1.5 to 4.7 kb led to a strong level of expression within muscle fibers. Surprisingly, quantitative analysis revealed that although expression of nlsLacZ was slightly higher in the postsynaptic sarcoplasm of muscle fibers, it was clearly not restricted to synaptic areas despite the presence of an N-box motif in the

promoter region shown recently by others (4, 5) and us (6, 7), to play a crucial role in directing the synapse-specific expression of AChR subunit genes and the utrophin gene, respectively. Nonetheless, deletion of 600 bp in intron 1 from the 1.5 kb promoter fragment completely abolished muscle expression. Transfection of motoneurons with these two latter constructs showed that both were effective in driving expression of LacZ in neuronal cells thereby indicating the presence of cis-acting elements essential for muscle expression in the first intron of the AChE gene. DNase 1 footprint analysis demonstrated that multiple regions in intron 1 were indeed protected by nuclear proteins extracted from skeletal muscles. Sequence analysis further showed that intron 1 contains a CArG box, as well as E- and N-box motifs. These results indicate that local transcriptional activation of the AChE gene is not the primary mechanism contributing to the maintenance of high levels of AChE transcripts at the neuromuscular junction. In addition, these results further highlight the role of DNA regulatory elements in the first intron of the AChE gene necessary for its expression in skeletal muscle.

In separate studies, we have examined the role of transcriptional and post-transcriptional regulatory mechanisms in the activity-linked regulation of AChE mRNA levels using the denervation model in both young (neo-natal to 2-week old) and mature rat skeletal muscles. Our results show that in comparison to the sharp increase (> 30-fold) in the levels of transcripts encoding the α -subunit of the acetylcholine receptor (AChR), denervation of adult muscle induced a rapid (within 2 days) and large (8-fold) decrease in the abundance of AChE mRNAs. Northern blot analysis also revealed that the two predominant species of AChE T transcripts expressed in adult muscle were reduced to a similar extent by muscle denervation. Furthermore, nuclear run-on assays showed that the transcriptional activity of the AChE gene remained essentially unchanged following denervation indicating that post-transcriptional events are responsible for the observed modifications in AChE transcript levels. In separate experiments, we examined whether denervation resulted in significant changes in the half-life of AChE and AChR α -subunit transcripts by injecting animals with actinomycin D. Quantitative analysis revealed that Egr-1 transcripts, used as a positive control in these experiments, were turning over rapidly (~1 h) in skeletal muscle. The estimated mRNA half-life in control muscle for AChE and the AChR α -subunit was ~ 9 and 6 h, respectively. Interestingly, denervation significantly increased the stability of both transcripts. In a final series of experiments, denervation of

muscle from young rats induced a parallel increase (3-fold) in the expression of AChE and AChR α -subunit mRNAs. Both increases resulted from an activation in the transcriptional activity of these genes as assessed by run-on analysis. Based on these findings, we also performed electrophoretic mobility shift assays (EMSA) using oligonucleotides corresponding to the CArG and N boxes located in the first intron and observed specific binding activities with nuclear protein extracts isolated from both young and adult rat skeletal muscles. Interestingly, more binding activities were detected in nuclear extracts obtained from young muscles as compared to those from adult tissues. In agreement with our nuclear run-on assays, additional EMSA also showed that binding activities to these DNA regulatory elements were dependent on the state of innervation of the muscle fibers since they both increased with denervation of young muscles.

Taken together, these findings indicate that following short-term denervation, expression of AChE mRNAs is differentially regulated in developing versus adult skeletal muscles. In addition, our results suggest that transcriptional regulatory mechanisms are crucial in the control of AChE transcript levels in muscles from young animals subjected to altered levels of neuromuscular activation. By contrast, post-transcriptional events appear as key regulatory steps in dictating AChE mRNA levels in adult muscles. Since neuromuscular junctions in young muscles still undergo several maturational steps, we suggest that the activity-dependent transcriptional regulation of the AChE gene demonstrates a high level of plasticity only during synapse formation and maturation. This work is supported by the Medical Research Council of Canada (MRC). F.A.M. and A.M.K. are supported by studentships from the Ontario Graduate Scholarship Program and the Rick Hansen Man in Motion Foundation. B.J.J. is a Scholar of the MRC.

References

- (1) Jasmin et al., *Neuron* 11: 467-477, 1993
- (2) Michel et al., *J. Cell Biol.* 127: 1061-1069, 1994
- (3) Sveistrup et al., *Am. J. Physiol. (Cell Physiol.)* 269: C856-C862, 1995
- (4) Koike et al., *Proc. Natl. Acad. Sci. USA* 92: 10624-10629, 1995
- (5) Duclert et al., *J. Biol. Chem.* 271: 17433-17438, 1996
- (6) Gramolini et al., *J. Biol. Chem.* 272: 8117-8120, 1997
- (7) Gramolini et al., *J. Biol. Chem.* 273: 736-743, 1998.

46

Cholinesterase activity as a biomarker of exposure to temephos in *Chironomus* sp. and *Nereis* (Hediste) *diversicolor* in Morbihan (Brittany, France) coastal wetlands

A. Jumel, L. Lagadic

Inra, Unité d'Ecotoxicologie Aquatique, Station de Recherche en Ichtyophysiologie, Biodiversité et Environnement (SCRIBE), Campus de Beaulieu, 35042 Rennes cedex, France

Cholinesterases (ChEs) have been widely used as biomarkers of exposure to organophosphates and carbamates in marine animals (Galgani and Bocquené, 1998). A pilot study has been undertaken to evaluate the ecological impact of Abate® (active ingredient temephos) used against mosquito larvae in Morbihan coastal wetlands. Midges *Chironomus* sp. and the marine worm *Nereis* (He-

diste) *diversicolor* were used as sentinel species in three pilot sites. In situ exposure to temephos was monitored in these animals using ChE activity.

Control and treated areas were defined in each pilot site. Sediment samples (4.5 L) were collected in both areas 24 h and 3 days after treatment. Water temperature, pH, dissolved oxygen

concentration and salinity were measured in sampling zones. Animals were isolated from the sediments and immediately frozen in liquid nitrogen. They were stored at -80°C until ChE activity measurement. Enzyme extracts were prepared from pools of 6 entire *Chironomus* sp. larvae and of the heads of 3 immature *N. diversicolor*. Individual measurements were performed on the head of mature *N. diversicolor*. Tissue samples were weighed and homogenized in 20 mM phosphate buffer pH 7.2, 0.1% Triton X-100, using an ice-cold glass homogenizer with a motor driven Teflon pestle. Homogenates were centrifuged at 10 000 g for 20 min at 4°C . The supernatants were used for ChE assay. ChE activity was determined according to Ellman et al. (1961) and Galgani and Bocquené (1998) using acetylthiocholine iodide (ACTC) as the substrate. Absorbance at 412 nm was recorded for 2 min, using a Uvikon 943 double-beam spectrophotometer (Kontron Instruments). The activity was corrected for non-enzymatic hydrolysis of the substrate and calculated using least-square linear regression analysis over the first 30 s of the kinetics. Protein contents were determined according to Bradford (1976), using BSA (fraction V) as the standard, and a commercial Coomassie Blue G-250 reagent solution (Pierce Chemical Co.). ChE activity was expressed as nmol of ACTC hydrolysed/min/mg protein.

ChE activities in control animals were 37.4 nmol ACTC hydrolysed/min/mg protein for midge larvae extracts, and 64.4 and 62.9 nmol ACTC hydrolysed/min/mg protein for the head extracts of immature and mature *N. diversicolor*, respectively. Basal levels of cholinesterase activity will be used as references for comparison to the animals exposed to temephos during the 1998 mosquito control campaigns.

This research was supported by the Conseil Général du Morbihan.

References

- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248–254.
- Ellman GL, Courtney KD, Andreas V JR, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7, 88–95.
- Galgani F, Bocquené G (1998) Biomarqueurs moléculaires d'exposition des organismes marins aux pesticides organophosphorés et carbamates. In: Lagadic, L., Caquet, Th., Amiard, J.-C. & Ramade, F. (eds.), *Utilisation de Biomarqueurs pour la Surveillance de la Qualité de l'Environnement*. Tec & Doc Lavoisier, Paris, 111–134.

Acetylcholinesterase inhibition by diazinon: A dual substrate model for kinetic study

M.A. Kamal, A.A. Al-Jafari

Department of Biochemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh, 11451, Saudi Arabia

Acetylcholinesterase (AChE) is a hydrolytic enzyme which controls the transmission of nerve impulses by hydrolyzing the acetylcholine (a neuro-transmitter) in the nervous system (Al-Jafari, et al., 1997). It is the target enzyme for testing toxicity induced by organophosphate and carbamate insecticides (Kamal, 1997). In the current study we have determined various kinetic parameters of AChE inhibition by diazinon, an organophosphate insecticide (Worek et al., 1997). The kinetic parameters obtained from figures 3 and 4 are as follows: $k_{oms}' = 0.821 \text{ min}^{-1}$; $K_{sms}' = 1.0 \text{ (mM min)}$; $k_{oms} = 0.378 \text{ min}^{-1}$; $K_{sms} = 0.40 \text{ (mM min)}^{-1}$; $K_{asms}' = 0.175 \text{ (mM/L)}^2$; $K_{asms} = 0.597 \text{ (L/mM)}^2$.

This is an unique, and a sensitive short approach. It is an promising approach for the estimation of various kinetic parameters for the inhibition of variety of enzymes by variety of chemicals, insecticides, herbicides and drugs.

References

- Al-Jafari AA, Kamal MA, Alhomid AS (1997) Thermodynamic investigation of camel retina acetylcholinesterase inhibition by cyclophosphamide. *J.Enz. Inhib.* 11, 275–283
- Kamal MA (1997) Investigation of the effect of lannate on kinetic parameters of acetyl-cholinesterase: Slightly concave mixed-type of inhibition system. *Biochem. Mol. Biol. Int.* 43, 1183–1193
- Worek F, Backer M, Thiermann H, Szinicz L, Mast U, Klimmek R, Eyer P (1997) Reappraisal of indications and limitations of oxime

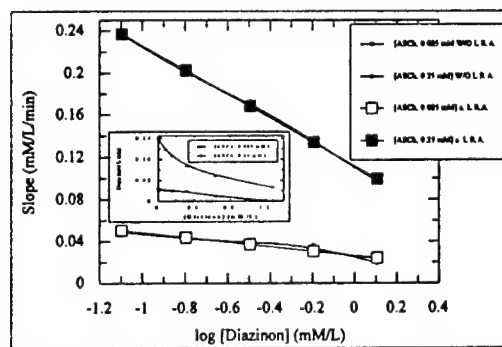


Figure 1. Secondary plot of figure: slope of each plot in figure 1 versus log concentration of diazinon at two concentrations of substrate (ASCh) as mentioned in legend box (W/O and a.L.R.A. means without and after linear regression analysis respectively). Inset: slope of each plot in figure 1 versus concentration of diazinon.

therapy in organophosphate poisoning. *Hum. Exp. Toxicol.* 16 (8), 466–472

Identification of inducible genes by activation of muscarinic acetylcholine receptors

H. von der Kammer, M. Mayhaus, C. Albrecht, B. Hoffmann, R.M. Nitsch

Center for Molecular Neurobiology, University of Hamburg, Hamburg, Germany

Muscarinic acetylcholine receptors (mAChR) are G protein-coupled cell surface receptors with classic seven transmembrane domain topology. Despite detailed knowledge of the molecular signaling pathways coupled to muscarinic receptors, little is known about genes and gene products that mediate the muscarinic receptor-associated functions. In brain, mAChRs are involved in long-term potentiation, synaptic plasticity, as well as in higher cognitive functions including learning and memory (Di Chiara et al., 1994). Such plastic alterations in neuronal structure and function are associated with rapid and transient transcription of activity-dependent genes like the immediate-early genes *c-fos*, *jun-B*, *Egr-1*, and *Egr-2* on attention, learning, memory and cognition (Huerta and Lisman, 1993). In addition to their physiological function, muscarinic receptors are involved in the pathological cholinergic deafferentation in Alzheimer's disease as well as in the posttranslational modification of the amyloid precursor protein (Nitsch et al., 1992).

In order to identify genes that are induced by mAChR, we developed a screening approach based on the mRNA differential display technique (Liang et al., 1994). Human HEK293 cells stably transfected with the muscarinic acetylcholine receptor subtype m1 were stimulated by carbachol, and identical cells generated in parallel were used as unstimulated controls. Cell culture experiments were performed in triplicates. Total RNA was prepared from the cells and transcribed to cDNA by using one-base-anchor primers HT₁₁A, HT₁₁G and HT₁₁C. The obtained cDNAs were subjected to polymerase chain reaction (PCR) employing the corresponding one-base-anchor oligonucleotides along with random primers by using PCR conditions as described (Zhao et al., 1995). PCR was performed in duplicates in the presence of α [³⁵S]-dATP along with dCTP, dTTP and dGTP. The PCR-products were separated on sequencing gels, gels were dried, and X-ray films were exposed. Differential bands were excised, the contained cDNAs were eluted and re-amplified by high-stringency PCR employing the corresponding composite primer pairs. Reamplified cDNAs

were purified and cloned into pBluescript IKS+. Cloned cDNA fragments were sequenced.

A set of 64 different random primers was generated with a HindIII restriction site followed by 7 bp random sequences which were specifically designed to ensure the statistically comprehensive analysis of all mRNA species in one cell population of a defined cell status.

After 68 of 192 possible PCR experiments, 34 differential bands were identified. Sequence analysis of 16 differential bands revealed that these bands corresponded to 8 different genes (table I).

By Northern blot analyses the expression of the immediate-early genes *egr-1*, *egr-2*, *egr-3*, NGFi-B, ETR101, and *c-jun* were proved to be upregulated by m1AChR activation. Time course analyses confirmed that mRNA levels of *Egr-1*, *Egr-2*, and *Egr-3* increased readily after m1AChR stimulation, and that a maximum was attained within 50 min. At that time, *Egr-4* mRNA was also detectable. Western blots and electromobility shift assays demonstrated synthesis of EGR-1 and EGR-3 proteins, as well as binding to DNA recognition sites in response to m1AChR activation. Activation of m1AChR increased transcription from EGR-dependent promoters including the acetylcholinesterase promoter. Activity-dependent regulation of *Egr-1* mRNA expression and EGR-1 protein synthesis was also observed in cells expressing m2, m3, or m4AChR subtypes (von der Kammer et al., 1998).

In addition, we identified hcyr61 as well as the previously unknown human gene *gig-2* (for G-protein coupled receptor induced gene), and verified muscarinic regulation of these genes by Northern blot analysis. hcyr61 encodes for a secretory protein with 381 amino acid residues. It is a member of the IGFBP (insulin-like growth factor binding protein) superfamily with possible roles in cellular interaction with the extracellular matrix. The cDNA of *gig-2* codes for a 372 amino acid protein and is has partial homologies with serine/threonine kinase domains. *Gig-2* is ubiquitously expressed, suggesting a housekeeping function that may be regulated in brain by neuronal activity.

Our results demonstrated that the developed screening system is useful to identify known genes as well as new genes that are under the control of m1AChR. First analyses revealed a high number of differential bands, with less than 10% false positive bands.

Table I. Characterisation of 16 analysed differential display bands of interest.

Gene product	No. of DD-bands	Verified regulation by mAChRs
Egr-1	3	+
Egr-2	1	+
Egr-3	3	+
Etr101	2	+
c-jun	3	+
NGFiB	1	+
gig-1 (hcyr61)	2	+
gig-2	1	+

References

- Di Chiara, G., Morelli, M., and Consolo, S. (1994) Trends Neurosci. 17, 228-233
- Huerta, P.T., and Lisman, J.E. (1993) Nature 364, 723-725
- Liang, P., Zhu, W., Zhang, X., Guo, Z., O'Connell, R.P., Averboukh, L., Wang, F., and Pardee, A.B. (1994) Nucleic Acids Res. 22, 5763-5764
- von der Kammer, H., Mayhaus, M., Albrecht, C., Enderich, J., Wegner, M., and Nitsch, R. (1998) J. Biol. Chem. in press
- Zhao, S., Ooi, S.L., and Pardee, A.B. (1995) Biotechniques 18, 842-850

49

Long-term modulations of cholinergic neurotransmission following chronic stress

D. Kaufer^a, A. Friedman^{a, b}, L. Pavlovsky^b, H. Soreq^a

^aDepartment of Biological Chemistry, Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem, Israel

^bDepartments of Physiology and Neurosurgery, Faculty of Health Sciences and, Zlotowski Center of Neuroscience, Beersheva, Israel

Acute and chronic stress are known to induce alterations in hippocampal structure (McEwen, 1997; Gurvits et al., 1996) as well as specific electrophysiological responses (Xu et al., 1997). We have recently shown that both acute psychological stress and exposure to anticholinesterases induce concerted calcium-dependent modulation of genes regulating acetylcholine availability. These changes suggested long-term decrease in the availability of acetylcholine in distinct brain regions and coincided with muscarinic-dependent rapid enhancement and delayed depression of neuronal excitability in the CA1 area of the hippocampus (Kaufer et al., 1998).

In order to study the molecular and physiological mechanisms bridging between the acute alterations in cholinergic neurotransmission and long-lasting modifications in hippocampal functions we tested the delayed consequences of repeated stress or anticholinesterase treatments on mice up to 6 weeks following 4 consecutive daily exposures. The CA1 synaptic responses were significantly suppressed and the sensitivity to muscarinic antagonists drastically enhanced in the treated mice. Moreover, the 'readthrough' splice variant of acetylcholinesterase mRNA accu-

mulated in perikarya and apical dendrites of pyramidal neurons in cortex and hippocampus. Together, these findings demonstrate weeks-long changes in cholinergic gene expression that are associated with long-lasting changes in cholinergic neurotransmission.

References

- Gurvits, T.V., Shenton, M.E., Hokama, H., Ohta, H., Lasko, N.B., Gilbertson, M.W., Orr, S.P., Kikinis, R., Jolesz, F.A., McCarley, R.W., Pitman, R.K. Magnetic resonance imaging study of hippocampal volume in chronic, combat-related posttraumatic stress disorder. *Biol.Psychiatry*. 40: 1091-1099 (1996).
- Kaufer, D., Friedman, A., Seidman, S. and Soreq, H. (1998). Acute stress facilitates long-lasting changes in cholinergic gene expression. *Nature*, in press.
- McEwen, B.S. Possible mechanisms for atrophy of the human hippocampus. *Mol.Psychiatry*. 2: 255-62 (1997).
- Xu, L., Anwyl, R., Rowan, M.J. Behavioural stress facilitates the induction of long-term depression in the hippocampus. *Nature*. 387: 497-500 (1997).

50

Mechanisms regulating expression of nicotinic acetylcholine receptor subunits

S.H. Keller, P. Taylor

Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093-0636, USA

Our investigative aim is to identify mechanisms which regulate expression of acetylcholine receptors at the cell surface. Monoclonal antibodies and high affinity ligands are available to detect expression of receptor subunits, and to distinguish whether subunits are folded or assembled. Furthermore, an extensive collection of published studies on degradation and assembly of the receptor, along with the selective ligands with which it interacts, make this system well suited to delineate the factors regulating expression of multisubunit transmembrane proteins. Subunits of the receptor assemble intracellularly to enclose a centralized cation channel, which is exported to the cell surface in the pentameric arrangement of subunits as α - γ - α - β - δ . Monomers and subunit dimers, $\alpha\delta$ and $\alpha\gamma$, are retained intracellularly, and assembly with the β -subunit is a requirement for export of the complex to the cell surface. Unassembled subunits show little accumulation in cells, compared to assembled complexes, indicating that mechanisms for degradation depend on subunit assembly. Subunits which accumulate in cells eventually become components of the mature receptor, pointing out that cellular factors which protect subunits from degradation contribute to maturation of the ion channel.

Our studies, with transiently expressed receptor subunits in mammalian culture cells, have identified that the chaperone protein, calnexin, and the conjugation of polyubiquitin chains to the subunit have a significant impact on subunit degradation, stability

and trafficking to the Golgi. Calnexin is a type-1 membrane protein, and it is thought to recognize oligosaccharide components in glycoproteins. By using the glycosidase inhibitor castanospermine, our experiments demonstrate that dissociating calnexin results in the enhanced polyubiquitination and degradation of receptor subunits in the proteasome. Our experiments also reveal that calnexin associates with unassembled subunits, and it dissociates when subunits assemble. In this manner, calnexin stabilizes subunits in preparation for assembly and expression at the surface. Since attachment of polyubiquitin molecules is thought to promote dislocation of proteins to the cytoplasm through the Sec61 translocon, one might hypothesize that attachment of polyubiquitin molecules acts to oppose transport of subunits into the secretory pathway leading to the Golgi. Therefore, calnexin attachment shows a role in the expression of subunits that are incorporated into the mature ion channel, by inhibiting polyubiquitination and dislocation to the cytoplasm.

Calnexin has an endoplasmic reticulum retention sequence in its cytoplasmic tail, and its attachment may also retain subunits in the endoplasmic reticulum. By using a cDNA which encodes for a truncated calnexin lacking the endoplasmic reticulum retention sequence, and the cell line, ts20, which expresses a temperature labile ubiquitin activating enzyme, retention in the endoplasmic reticulum and polyubiquitination can be inhibited.

Proteins expressed in ts20 cells, at 40 °C, are not polyubiquitinated. Alpha-subunits can be induced to be transported to the Golgi when coexpressed with truncated calnexin in ts20 cells at 40 °C, as detected by confocal microscopy. In the absence of these reagents, for example, when expression occurs in HEK cells, α -subunits remain in the endoplasmic reticulum. Alpha-subunits expressed in ts20 cells without coexpression of truncated calnexin, are also sequestered in the endoplasmic reticulum. Calnexin is not associated with assembled subunits, indicating that its role for retaining subunits in the endoplasmic reticulum is precluded when subunits assemble. These observations indicate that calnexin may have dual roles: along with inhibiting dislocation into the ubiquitin-proteasome pathway,

calnexin may sequester receptor subunits in the endoplasmic reticulum prior to subunit assembly. The outcome of both of these roles is to stabilize unassembled subunits in place in the endoplasmic reticulum, thereby enhancing opportunities for assembly with other subunits of the receptor. In summary, our findings indicate that chaperone protein attachment and ubiquitination act together to regulate the fate of the unassembled subunit pool, a part of which is eventually incorporated into the mature ion channel. Observations that emerge from studies on the acetylcholine receptor should be applicable to the expression of other multisubunit transmembrane proteins. Supported by an American Heart Association postdoctoral fellowship to S.H.K. and NIH Grant GM 18360 to P.T.

51

nAChR and α -neurotoxin: New tools for old acquaintances

P. Kessler, S. Maurin, A. Ménez

CEA/Saclay, Département d'Ingénierie et d'Etudes des Protéines, 91191 Gif-sur-Yvette, France

Despite numerous studies (Oswald et al., 1982, Trémeau et al., 1995, Machold et al., 1995, Ackermann and Taylor, 1997), the structure of complexes between a snake curaremimetic toxin and nicotinic acetylcholine receptors (nAChR) remains unclear. This study, therefore, aimed at bringing new insights on this old question by exploiting today's techniques.

Using solid phase peptide synthesis, we prepared large amounts of various mutants of *Naja nigricollis* α -toxin. Two toxin mutants, R33C and K47C, were synthesized and characterized. These mutations caused moderate affinity decreases for *Torpedo* receptor.

Previous photoaffinity experiments done on nAChR showed that an aryldiazonium ion, DDF, as developed by Goeldner, Hirth and colleagues (Langenbuch-Cachat et al., 1988), is a well suited photoactivatable probe which allows both high coupling yields and labelling of several functionally important receptor residues. We therefore synthesized an analog of DDF, and coupled it to the free cysteine of R33C and K47C mutants, through a cleavable disulfide bond. Photoaffinity labelling experiments carried out with R33C-DDF toxin analogs (see figure 1), showed an efficient incorporation of the probe in the *Torpedo* α subunit only, indicating the proximity of the second loop of the toxin with this subunit. Synthesis of a radioactive DDF analog is in progress, to identify the residues labelled on the receptor.

Treatment of nAChR with low concentration of DTT is known to selectively reduce the α -subunit disulfide bond Cys192-Cys193, which is located in the acetylcholine binding site. Thus, the cysteines 192 or 193 can be labelled with small antagonists such as MBTA (Kao and Karlin, 1986). We explored the possibility that a maleimido analog introduced at an appropriate position of the toxin (see figure 1) could also label specifically Cys192 or Cys193. Using different R33C toxin analogs, we indeed observed an efficient labelling of the reduced cysteinyl residues. This result not only agrees with our photoaffinity experiments made with R33C-DDF that R33 interacts with the α -subunit but demonstrates its proximity with the acetylcholine binding site. By contrast, K47

is not in such a close proximity to the α -subunit, since K47 toxin analogs unexpectedly labelled the δ subunit. The only disulfide bond that can be reduced under our conditions in this subunit, involves the δ - δ bond that links two *Torpedo* receptor monomers. Consequently, we suggest that the K47 residue on the toxin third loop is in proximity to the penultimate C500 residue of the nAChR δ subunit.

These preliminary results demonstrate the interaction of the central toxic loop with the acetylcholine binding site on the α subunit, and of the third loop with the COOH terminus part of the δ subunit, in agreement with a location of the binding site between two subunits (as shown here for α and δ). If labelling of the d-C500 residue would be confirmed, this would imply an interaction of the toxin with the outside face of the extracellular part of the receptor.

References

- Oswald, R, Changeux, J-P (1982) Crosslinking of α -bungarotoxin to the acetylcholine receptor from *Torpedo marmorata* by ultraviolet light irradiation. FEBS Letters 139, 225-229
- Trémeau, O, Lemaire, C, Drevet, P, Pinkasfeld, S, Ducancel, F, Boulain, J-C, Ménez, A (1995) Genetic engineering of snake toxins. J. Biol. Chem. 270, 9362-9369
- Machold, J, Utkin, Y, Kirsch, D, Kaufmann, R, Tsetlin, V, Hucho, F (1995) Photolabeling reveals the proximity of the α -neurotoxin binding site to the M2 helix of the ion channel in the nicotinic acetylcholine receptor. Proc. Natl. Acad. Sci. USA 92, 7282-7286
- Ackermann, E, Taylor, P (1997) Nonidentity of the α -neurotoxin binding sites on the nicotinic acetylcholine receptor revealed by modification in α -neurotoxin and receptor structures. Biochemistry 36, 12836-12844
- Langenbuch-Cachat, J, Bon, C, Mülle, C, Goeldner, M, Hirth, C, Changeux, J-P (1988) Photoaffinity labeling of the acetylcholine binding site on the nicotinic receptor by an aryldiazonium derivative. Biochemistry 27, 2337-2345
- Kao, P and Karlin, A (1986) Acetylcholine receptor binding site contains a disulfide crosslink between adjacent half cystinyl residues. J. Biol. Chem. 261, 8085-8088

The role of charged transmembrane residues of rVAcHT on ACh transport and vesamicol binding

M.-H. Kim^a, M. Lu^a, E.-J. Lim^b, Y.-G. Chai^b, L.B. Hersh^a

^aDept. of Biochemistry, University of Kentucky, Lexington, KY 40536, USA

^bDept. of Biochemistry, Hanyang University, Ansan, Korea

The neurotransmitter acetylcholine (ACh) is synthesized in the cytoplasm of cholinergic neurons and transported into synaptic vesicles by the vesicular acetylcholine transporter (VAcHT). Rat VAcHT has several charged amino acid residues within its predicted transmembrane domains (TM) which may play important roles in structure and function. In order to identify the functional charged residues of rVAcHT involved in the transport of acetylcholine and in vesamicol binding, site directed mutagenesis of rVAcHT was undertaken. Changing Asp46 (D46N) in TM2, Asp225 (D225N) in TM6, and His444 (H444R) in TM12 respectively, had no effect on ACh transport or vesamicol binding. However, replacement of His338 in TM8 with Arg or Asp398 in TM10 with Asn completely eliminated both ACh transport and vesamicol binding. Surprisingly, mutant D193N in TM4 did not affect ACh transport activity; however vesamicol binding was dramatically reduced. The same loss of vesamicol binding without affecting ACh transport was also seen with two other mutants; H413R mutant which is in the loop between TM10 and 11 and E449Q in TM12. Interestingly, with mutants K131A in TM2 or D425N in TM11, transport activity for ACh was completely blocked without any change in vesamicol binding. These mutants as well as mutant D193N, H413R, and E449Q clearly dissociate ACh binding and transport from vesamicol binding. These data suggest that Lys131 in TM2, His338 in TM8, Asp398 in TM10 and Asp425 in TM11 are important to ACh binding and transport. Asp193 and Asp398 in TM4 and TM10 respectively, Glu449 in TM12, His338 in TM8, and His413 in the cytoplasmic loop between TM10 and TM11 are involved in vesamicol binding. Together these studies begin to unravel the topology of the vesicular acetylcholine transporter.

References

- 1 Bahr, B. A., Clarkson, E. D., Rogers, G. A., Noremborg, K., and Parsons, S. M. (1992) A kinetics and allosteric model for the acetylcholine transporter-vesamicol receptor in synaptic vesicles. *Biochemistry* 31, 5752-5762
- 2 Erickson, J. D., Varoqui, H., Schafer, M. K. H., Modi, W., Diebler, M. F., Weihe, E., Rand, J. B., Eiden, L. E., Bonner, T. I., and Usdin, T.B. (1994) Functional identification of a vesicular acetylcholine transporter and its expression from a "cholinergic" gene locus. *J. Biol. Chem.* 269, 21929-21932.
- 3 Finn, J. III. and Edwards, R. H. (1997) Individual residues contribute to multiple differences in ligand recognition between vesicular monoamine transporters 1 and 2. *J. Biol. Chem.* 272, 16301-16307.
- 4 Finn, J. P. III, Edwards, R. H. (1998). Multiple residues contribute independently to differences in ligand recognition between vesicular monoamine transporters 1 and 2. *J. Biol. Chem.* 273, 3943-3947
- 5 Liu, Y., Edwards, R. H. (1997). Differential localization of vesicular acetylcholine and monoamine transporters in PC12 cells but not CHO Cells. *J. Cell Biol.* 139, 907-916
- 6 Liu, Y., Edwards, R. H. (1997). The role of vesicular transport proteins in synaptic transmission and neural degeneration.. *Annu. Rev. Neurosci.* 20, 125-156
- 7 Merickel, A., Rosandich, P., Peter, D., and Edwards, R. H. (1995) Identification of residues involved in substrate recognition by a vesicular monoamine transporter. *J. Biol. Chem.* 270, 25798-25804.
- 8 Merickel, A., Kaback, H. R., Edwards, R. H. (1997). Charged residues in transmembrane domains II and XI of a vesicular monoamine transporter form a charge pair that promotes high affinity substrate recognition. *J. Biol. Chem.* 272, 5403-5408.
- 9 Nyuyen, M.L. and Parsons, S. M. (1995) Effects of internal pH on the acetylcholine transporter of synaptic vesicles. *J. Neurochem.* 64,1137-1142
- 10 Peter, D., Vu, T., Edwards, R. H. (1996). Chimeric vesicular monoamine transporters identify structural domains that influence substrate affinity and sensitivity to tetraabenazine. *J. Biol. Chem.* 271, 2979-2986.
- 11 Shirvan, A., Laskar, O., Steiner-Mordoch, S. and Schuldiner, S. (1994) Histidine-419 plays a role in energy coupling in the vesicular monoamine transporter from rat. *FEBS Lett.* 356, 145-150
- 12 Song, H.J., Ming, G. L., Fon, E., Bellocchio, E., Edwards, R. H., and Poo, M. M. (1997) Expression of a putative vesicular acetylcholine transporter facilitates quantal transmitter packaging. *Neuron* 18, 815-826.
- 13 Suchi, R., Stern-Bach, Y. and Schuldiner S. (1992) Modification of arginyl or histidyl groups affects the energy coupling of the amine transporter. *Biochemistry* 31, 12500-12503
- 14 Varoqui, H. and Erickson, J. D. (1996). Active transport of acetylcholine by the human vesicular acetylcholine transporter. *J. Biol. Chem.* 271, 27229-27232.
- 15 Varoqui, H. and Erickson, J. D. (1997) Vesicular neurotransmitter transporters, Potential sites for the regulation of synaptic function. *Mol. Neurobiol.* 15, 165-192.
- 16 Varoqui, H., Erickson, J. D. (1998). The cytoplasmic tail of the vesicular acetylcholine transporter contains a synaptic vesicle targeting signal. *J. Biol. Chem.* 273, 9094-9098.

53

Ca²⁺-dependent regulation by calcitonin gene-related peptide of neuronal type nicotinic acetylcholine receptor in neuromuscular pre- and postsynapse

I. Kimura, K. Dezaki

Department of Chemical Pharmacology, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan

Calcitonin gene-related peptide (CGRP), the major neuropeptide, coexists with acetylcholine (ACh) at the motor nerve endings (Takami et al., 1985a, b; Matteoli et al., 1988), is released by electrical nerve stimulation or by cholinesterase inhibition in intact skeletal muscle, and the CGRP may negatively modulate nerve stimulation-evoked ACh release (Kimura et al., 1997) (figure 1). CGRP binds to its receptors (R) located at the neuromuscular postsynapse (Jennings and Mudge, 1989; Poper and Micevych, 1989; Roa and Changeux, 1991). This peptide prolongs open time of nicotinic AChR channel currents (Lu et al., 1993; Owens and Kullberg, 1993) in cultured myotubes and regulates neuromuscular transmission by modulation of nicotinic AChR phosphorylation and desensitization (Miles et al., 1989; Dezaki et al., 1996). CGRP prolongs the duration of nerve-stimulated non-contractile slow Ca²⁺ mobilization and CGRP₈₋₃₇, a CGRP antagonist, shortens its duration (Kimura et al., 1993). Confocal imaging of flexor digitorum brevis (FDB) muscle cells indicated that bath-application of CGRP after ACh application potentiated the ACh-elicited

slow Ca²⁺ signal. Cotreatment of muscle cells with ACh plus CGRP led to a synergistic enhancement in the slow Ca²⁺ response compared with those elicited by ACh alone (figure 1). The first brief and localized rise in the [Ca²⁺]_i appeared to be due to the effect of CGRP alone. CGRP pretreatment, not after ACh application, has no effect on the ACh-elicited Ca²⁺ signals. The nerve-stimulated non-contractile Ca²⁺ mobilization is specially blocked by competitive nicotinic antagonists such as (+)-tubocurarine and pancuronium at low concentrations that have no effect on contractile Ca²⁺ transients (Kimura et al., 1990). ACh responses were completely depressed in both components and the potentiation by CGRP was greatly depressed in the presence of (+)-tubocurarine. The involvement of the voltage-sensitive L-type Ca²⁺ channels in the CGRP potentiating mechanism may be ruled out because a Ca²⁺ channel blocker nitrendipine did affect neither ACh responses nor CGRP-induced potentiation. Therefore, CGRP may activate its own receptor, then indirectly enhance the neuronal type nicotinic AChR-operated slow Ca²⁺ signal because CGRP does

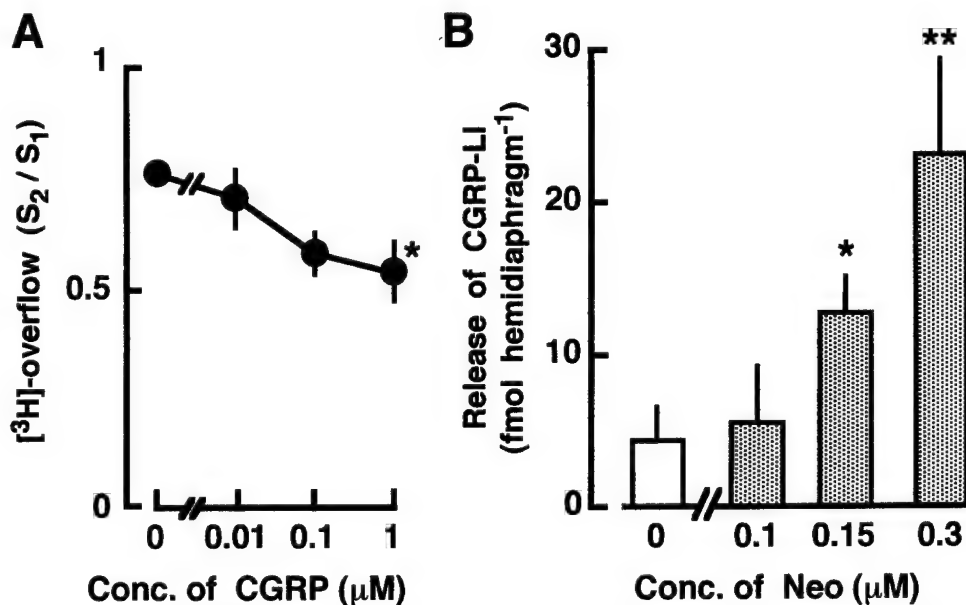


Figure 1. A. Concentration-response curve of CGRP for electrically evoked [³H]ACh release (S₂/S₁, the ratio of the value at two stimulation periods) from mouse phrenic nerve-diaphragm muscle preparations. CGRP was applied in various concentrations 15 min before S₂. Each point represents the mean value for relative ratios of ACh release (S₂/S₁) (*n* = 3–5) and vertical bars represent S.E.M. Significant difference from the control (without drug) was analyzed by one-way analysis of variance followed by Scheffe's test (**P* < 0.05). B. Increasing effect of neostigmine on resting release of CGRP-like immunoreactivity (CGRP-LI) from rat phrenic nerve-hemidiaphragm muscle preparations. The tissues were incubated for 10 min with (0.1, 0.15 and 0.3 μM; stippled columns) or without (open column) neostigmine after equilibration. Significant differences from the control (without drug) were analyzed by one-way analysis of variance followed by Scheffe's test (**P* < 0.05 and ***P* < 0.01). Values are means ± S.E.M. of 3 to 7 experiments.

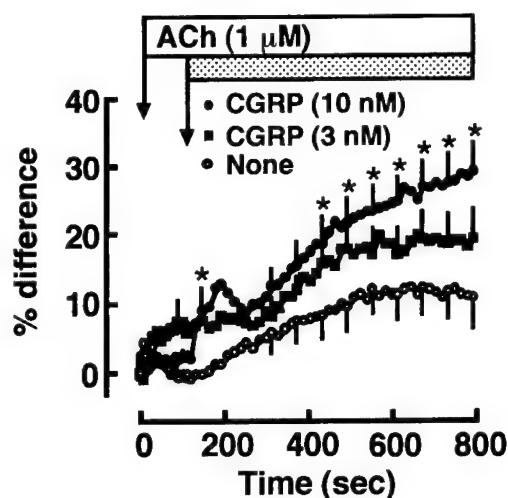


Figure 2. Potentiating effect of CGRP on ACh (1 μ M)-elicited slow Ca^{2+} component in mouse FDB single skeletal muscle cells. The cells were exposed to CGRP 2 min after ACh application. The percentage difference of the fluorescence intensity between inside and outside the endplate region was estimated. Data are expressed as means \pm S.E.M. ($n = 27-30$). * $P < 0.05$; significant differences from control response with ACh alone based on one-way ANOVA followed by Scheffé's test.

not activate directly muscle type nicotinic AChR at the muscle surface (Takami et al., 1985b).

References

- Kimura I, Tsuneki H, Dezaki K, Kimura M (1993) Enhancement by CGRP of nicotinic receptor-operated noncontractile Ca^{2+} mobilization at the mouse neuromuscular junction. *Br J Pharmacol* 110, 639-644
- Dezaki K, Kimura I, Tsuneki H, Kimura M (1996) Enhancement by CGRP of noncontractile Ca^{2+} -induced nicotinic receptor desensitization at the mouse neuromuscular junction. *Br J Pharmacol* 118, 1971-1976
- Jennings C G B, Mudge A W (1989) Chick myotubes in culture express high-affinity receptors for CGRP. *Brain Res* 504, 199-205
- Kimura I, Kondoh T, Kimura M (1970) Postsynaptic nicotinic ACh receptor-operated Ca^{2+} transients with neostigmine in phrenic nerve-diaphragm muscle of mice. *Brain Res* 507, 309-311
- Kimura I, Okazaki M, Nojima H (1997) Mutual dependence of CGRP and ACh release in neuromuscular preparations. *Eur J Pharmacol* 330, 123-128
- Lu B, Fu W, Greengard P and Poo M-M (1993) CGRP potentiates synaptic responses at developing neuromuscular junction. *Nature* 363, 76-79
- Matteoli M, Haimann C, Torri-Tarelli F, Polak J M, Ceccarelli B and De Camilli P (1988) Differential effect of α -latrotoxin on exocytosis from small synaptic vesicles and from large dense-core vesicles containing CGRP at the frog neuromuscular junction. *Proc Nat Acad Sci USA*, 85, 7366-7370
- Miles K, Greengard P, Haganir R L (1989) CGRP regulates phosphorylation of the nicotinic AChR in rat myotubes. *Neuron* 2, 1517-1524
- Owens J L, Kullberg R W (1993) CGRP lengthens AChR channel open time in developing muscle. *Receptors and Channels* 1, 165-171
- Poper P and Micevych P E (1989) Localization of CGRP and its receptors in a striated muscle. *Brain Res* 496, 180-186
- Roa M and Changeux J-P (1991) Characterization and developmental evolution of a high-affinity binding site for CGRP on chick skeletal muscle membrane. *Neurosci* 41, 563-570
- Takami K, Kawai Y, Shiosaka S, Lee Y, Giris S, Hillyard C J, MacIntyre I, Emson P C and Tohyama M (1985a) Immunohistochemical evidence for the coexistence of CGRP and choline acetyltransferase-like immunoreactivity in neurons of the rat hypoglossal, facial and ambiguous nuclei. *Brain Res* 328, 386-389
- Takami K, Kawai Y, Uchida S, Tohyama M, Shiotani Y, Yoshida H, Emson P C, Giris S, Hillyard C J and MacIntyre I (1985b) Effect of CGRP on contraction of striated muscle in the mouse. *Neurosci Lett* 60, 227-230

The Schwann cell at the neuromuscular junction

J. Koenig^{a, b}, S. De La Porte^c, J. Chapron^{b, d}

^aInstitut de Myologie, Groupe Hospitalier Pitié-Salpêtrière, 47, boulevard de l'Hôpital, F-75651 Paris cedex 13, France

^bLaboratoire de Neurobiologie Cellulaire, Université de Bordeaux II, Avenue des Facultés, 33405 Talence cedex, France

^cLaboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS UPR 9040, Avenue de la Terrasse, 91198 Gif-sur-Yvette cedex, France

^dURA-CNRS 1126, 33120 Arcachon, UMR 5807, 33405 Talence, France

Schwann cells wrap around nerve axons to provide electrical insulation and are essential for the formation, maintenance and repair of the neuromuscular junction (NMJ) (Balice-Gordon, 1996). At the NMJ, they are juxtaposed with nerve terminal and muscle cell, with which they interact during synaptogenesis. We focused on their role in maturation of the NMJ.

Establishment of an NMJ involves a sequence of complex mechanisms. The progression of events, from the first neuromuscular contact (14-15 embryonic days) to the mature synapse (15 days post-natal), occurs over 3 weeks. As soon as the first nerve-muscle contact is established, acetylcholine receptors (AChR) and cholinesterases (acetylcholinesterase [AChE] and butyrylcholinesterase [BChE]) accumulate at synaptic sites. The accumulation of AChR in the muscular membrane is a prerequisite for normal accumulation of AChE in the basal lamina of the synaptic cleft (De La Porte et al., 1998). AChR mature up to 2 weeks after

birth under the control of molecules synthesised by the motoneuron. AChR consists of α , β , γ and δ subunits at the embryonic stage, and the γ subunit is replaced by the ϵ subunit 2-3 days after innervation. This substitution affects the channel properties of the AChR (conductance and open time). Two molecules synthesised by the motoneuron are known to induce expression of the ϵ gene: acetylcholine receptor-inducing activity (ARIA)/heregulin and agrin. Another neuronal molecule, calcitonin-gene related peptide (CGRP), also increases transcription of the AChR α -subunit gene, but not of other subunits. Agrin also induces the clustering of AChR, AChE, BChE and heparan sulphate proteoglycan (HSPG), a component of the basal lamina.

Cholinesterases exist in several oligomeric molecular forms, distinguished by their sedimentation coefficient: the globular and the asymmetric forms. The catalytic subunits corresponding to AChE and BChE are encoded by distinct genes. The role of AChE

in cholinergic transmission is unambiguous, but the function of BChE is not clear except as an adhesion molecule. BChE, which is little expressed in the adult NMJ, also hydrolyses the neurotransmitter acetylcholine, and may thus participate in neurotransmission *in vitro*, and *in vivo* during development, and to a lesser degree in the adult NMJ. AChE and BChE are initially present in equal amounts at the NMJ, but as we demonstrated BChE levels diminish sharply between 7 and 15 days after birth, the stage at which the synaptic Schwann cell membrane becomes juxtaposed with the muscle membrane (Desaky and Uehara, 1987).

Synapses obtained *in vitro*, by conventional techniques of contact between myoblasts and spinal cord neurons, have the morphological, biochemical and electrophysiological characteristics of embryonic synapses. The events of postnatal maturation, such as disappearance of polyinnervation, changes in levels of AChE and BChE, and appearance of junctional folds, have never been observed, whatever the duration of culture or the age (embryonic or adult) of the cells used. In co-cultures of adult human muscles with explants from whole cord and dorsal root ganglia of 13- to 14-day-old rat embryos, Kobayashi et al. (1987) observed the formation of mature muscle fibres, as characterised by contractions and striations, well-organised AChE clusters, and the trend from multifocal to unifocal innervation. This result suggested to us that Schwann cells of dorsal root ganglia participate in synaptic maturation.

The factors involved in the morphological maturation (disappearance of polyinnervation, appearance of junctional folds) and changes of synaptic cholinesterases were not identified and we

postulated that Schwann cells were implicated in these events. We prepared mixed cultures, combining muscle cells (from the hindleg of 18-day-old rat embryos), spinal cord cells (from 14-day-old embryos) and Schwann cells (from the sciatic nerve of newborn animals or from the Schwann cell line TSC2, a gift from Prof. Tennekoon, Philadelphia, USA). Schwann cells and spinal cord cells were added to the muscle cells at day 5 of culture, the stage at which the myotubes were formed, contracting and morphologically differentiated. We used a monoclonal antibody (6.17) obtained in the laboratory, which recognises a molecule synthesised by Schwann cells and co-localised with the AChR at the NMJ. 6.17 antigen appears to be localised in the synaptic space, but not in the basal lamina. *In vivo* in the rat, 6.17 antigen is initially distributed diffusely and concentrates at the synapse 15 days after birth, and its maintenance at the NMJ depends on innervation (Koenig et al., 1988).

We have shown that during *in vitro* synaptogenesis, the addition of Schwann cells to muscle-neuron co-cultures: 1) allows visualisation of antigen 6.17 at the synapse and induces synaptic maturation (decrease in number of synapses and appearance of junctional folds (Chapron and Koenig, 1989); and 2) induces the disappearance of BChE at synaptic clusters, leaving only AChE activity, as in the adult neuromuscular junction (Chapron et al., 1997)). This maturation is inhibited by the presence of antibody 6.17. Antigen 6.17 is secreted by Schwann cells and TSC2, since medium conditioned by these cells reproduced the effect of the cells themselves. It may be wondered whether antigen 6.17 corresponds to a previously characterised molecule (cf. Chapron et al., 1997), and its molecular characterisation is in progress (in collaboration with H. Chneiweiss, INSERM U114, Paris). These results prompted us to postulate that Schwann cells could mediate changes in synaptic maturation during development. Heregulins and their specific receptors, members of the erbB family of tyrosine kinases, have been implicated in the control of growth and development of Schwann cells. Homozygous erbB3 mutant embryos lack Schwann-cell precursors and Schwann cells that accompany peripheral axons of motor (and sensory) neurons (Reithmacher et al., 1997). It will be of interest to look at the possible presence of high levels of BChE in such mutants.

AChR clustering may be induced by neuronal agrin, electric fields, latex beads, and basal lamina components (collagen, laminin). As the Schwann cell is closely associated with the nerve terminal, we investigated whether it plays a role in the formation of AChR clusters, and found that it does. When primary Schwann cells or medium conditioned by Schwann cells or by TSC2 cells, were added to muscle cells in culture, the number of AChR sites increased by only 10%, but the number of AChR clusters increased by 100% (figure 1). Antibody 6.17 does not inhibit the Schwann cell-mediated increase in the number of AChR clusters. This strengthens the idea that antigen 6.17 is specifically implicated in synaptic maturation. We have tested the possibility that agrin synthesised by the Schwann cell is the molecule responsible for this increase. But the agrin isoform expressed by the Schwann cell is the inactive one (B0) (in collaboration with G. Escher, Institut d'Embryologie, Lausanne, Switzerland). We are currently testing different factors synthesised and released by Schwann cells.

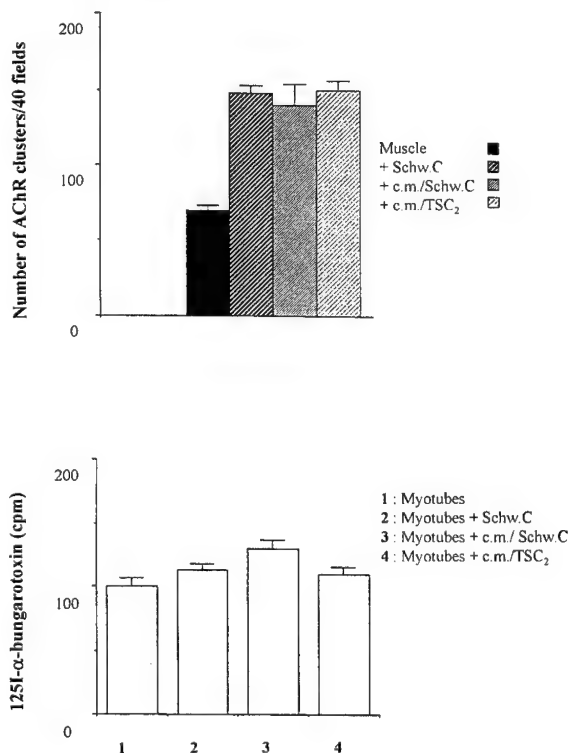


Figure 1. Number of AChR clusters (top) and ^{125}I - α -bungarotoxin binding sites (bottom) in 7-day-old myotubes cultured for 2 days with Schwann cells or with conditioned medium (c.m.) by Schwann or TSC2 cells.

References

- Balice-Gordon RJ (1996) Dynamic roles at the neuromuscular junction. *Curr. Biol.* 6, 1054-1056.

- Chapron J, Koenig J (1989) In vitro synaptic maturation. *Neurosci. Lett.* 106, 19–22.
- Chapron J, De La Porte S, Delépine L, Koenig J (1997) Schwann cells modify expression of acetylcholinesterase and butyrylcholinesterase at rat neuromuscular junctions. *Eur. J. Neurosci.* 9, 260–270.
- De La Porte S, Chaubourt E, Fabre F, Poulas K, Chapron J, Eymard B, Tzartos S, Koenig J (1998) Accumulation of acetylcholine receptors is a necessary condition for normal accumulation of acetylcholinesterase during in vitro neuromuscular synaptogenesis. *Eur. J. Neurosci.* 10, in press.
- Desaky J, Uehara Y (1987) Formation and maturation of subneural apparatus at neuromuscular junctions in post-natal rats. A scanning and transmission electron microscopic study. *Dev. Biol.* 199, 390–401.
- Koenig J, Chapron J, Vigny M (1988) Does the Schwann cell synthesize a molecule concentrated at the neuromuscular junction? *Neurosci. Lett.* 89, 265–270.
- Riethmacher D, Sonnenberg-Riethmacher E, Brinkmann V, Yamaai T, Lewin GR, Birchmeier C (1997) Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* 389, 725–730.

55

Recovery of brain acetylcholinesterase activity in common carp *Cyprinus carpio* L. exposed to chlorfenvinphos and carbofuran insecticides

D. Korami, H. Eric

Unité de zoologies générale et appliquée, Faculté Universitaire des Sciences Agronomique de Gembloux, 2, passage des déportés B-5030 Gembloux, Belgium

Organophosphates and carbamates used in intensive agricultural production can reach naturel water either via sepage of chemicals from the soil or directly due to the spraying against pests. These compounds may become concentrated in organs of aquatic organisms, especially those at the top of the food chain. The main mechanism of action of these organophosphates and carbamate insecticides involves the inhibition of acetylcholinesterase (AChE). However, if exposure to the inhibitor is discontinued, AChE activities could recover the normal level.

In this work successive extraction of AChE of the common carp brain showed 85.6% of soluble form against 14.4% of membrane bound form.

After 96 h of exposure of 6–8 cm length carp to chlorfenvinphos (2.1×10^{-4} mg/L) and carbofuran 3×10^{-3} mg/L, the AChE activity decreases respectively to 10% and 70% compared to control group. Three times higher (15 days) the exposition time (96 h) is necessary, after transfer in clean water, to recover the AChE activity level identical to that of control.

56

Laser-assisted cell-picking and subsequent RT-PCR allows subtype analysis of mRNAs derived from the cholinergic gene locus in rat cardiac neurons

W. Kummer^a, L. Fink^{b,c}, M. Dvorakova^a, R. Haberberger^a, R.M. Bohle^c

^aInstitute for Anatomy and Cell Biology, ^bDepartment of Internal Medicine, and ^cInstitute for Pathology, Justus-Liebig-University, 35385 Giessen, Germany

Choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (VACHT) are encoded by the 'cholinergic gene locus' in a peculiar nested fashion, in that the entire coding region of VACHT mRNA is located between the first non-coding exons ('R' and 'N') of the ChAT gene (Usdin et al., 1995). A third non-coding exon ('M') of the ChAT gene follows between the N-exon and the coding ChAT exons. In the rat, a remarkable variety of mRNA species is derived from this cholinergic gene locus: transcription start initiated from three different promoters results in R-, M- and N-type ChAT mRNAs from which subtypes R1/R2 and N1/N2 are the result of differential splicing. The R-exon is also contained in V1a- and V1b-type VACHT mRNA whereas the V2-type VACHT mRNA is coded for in one exon. In cultured sympathetic postganglionic neurons all types of ChAT and VACHT mRNAs can be induced and their ChAT and VACHT expressions are coordinately but differentially regulated (Misawa et al., 1995). Extracts from whole rat spinal cord contain all five species of ChAT mRNAs (Kengaku et al., 1993), but it is not known whether individual mRNA species are cell-specifically expressed. A tool for subtype analysis of mRNA species derived from the cholinergic gene locus in select neuronal populations in situ is desirable for solving this and related questions, but yet has not been available. Focusing upon intrinsic cardiac neurons of the rat we addressed this issue by laser-assisted cell-picking (Schütze and Clement-Sengewald, 1994) and subsequent RT-PCR.

Rat heart atria were quick-frozen in melting isopentane, cryosections (6 µm) were collected on coverslips and stained with hematoxylin. Utilizing a nitrogen laser microbeam (337 nm wavelength; P.A.L.M., Wolfrahtshausen, Germany) connected to an inverted microscope, identified neuronal section profiles were dissected by photolysis from surrounding tissue under microscopic control and harvested with a micromanipulator. Cardiomyocytes obtained from the same sections served as control. From 1 to 11 neuronal section profiles were pooled and processed as a single specimen. They were transferred into 10 µL of a modified first strand buffer and snap frozen. Before cDNA synthesis, tubes were heated and the content divided into two aliquots of equal volume. One of them was subjected to complete RT-PCR, the other was processed identically except that reverse transcriptase was omitted. Thus, for each individual sample an internal control for un-

nergetic gene locus in select neuronal populations in situ is desirable for solving this and related questions, but yet has not been available. Focusing upon intrinsic cardiac neurons of the rat we addressed this issue by laser-assisted cell-picking (Schütze and Clement-Sengewald, 1994) and subsequent RT-PCR.

Rat heart atria were quick-frozen in melting isopentane, cryosections (6 µm) were collected on coverslips and stained with hematoxylin. Utilizing a nitrogen laser microbeam (337 nm wavelength; P.A.L.M., Wolfrahtshausen, Germany) connected to an inverted microscope, identified neuronal section profiles were dissected by photolysis from surrounding tissue under microscopic control and harvested with a micromanipulator. Cardiomyocytes obtained from the same sections served as control. From 1 to 11 neuronal section profiles were pooled and processed as a single specimen. They were transferred into 10 µL of a modified first strand buffer and snap frozen. Before cDNA synthesis, tubes were heated and the content divided into two aliquots of equal volume. One of them was subjected to complete RT-PCR, the other was processed identically except that reverse transcriptase was omitted. Thus, for each individual sample an internal control for un-

warranted amplification of genomic DNA fragments was run. Two primer pairs for amplification of ChAT mRNA were used: one of them was intron-spanning and located in the coding region of ChAT, the other was specific for that region of the non-coding R-exon which codes only for R1-type mRNA. Hence, the latter primer pair was not intron-spanning.

Using the intron-spanning primer pair, ChAT mRNA could be detected in neuronal samples by RT-PCR, and amplicons derived from cDNA were readily discriminated from those derived from genomic DNA in agarose gels simply by molecular size. Thus, the general feasibility of the technique was demonstrated, but these experiments did not allow subtype analysis of mRNA species since all diversity lies in the 5' non-coding region. Utilizing the R1-type specific primer pair, in initial experiments glial cells including their nuclei were not thoroughly separated from neuronal section profiles. From these samples an amplicon of expected size (121 bp) was generated by RT-PCR, but also when reverse transcriptase was omitted. Thus, in the present technique, which omits a DNase digestion step, contaminating DNA will also serve as a template in the following PCR. Therefore, in order to minimize load of genomic DNA in the sample, microscopically visible nuclei were photolyzed by the UV laser in subsequent experiments. Under these conditions, a single amplicon of expected size was generated by RT-PCR in 56% of neuronal samples, but only in 9% of samples when reverse transcriptase was omitted. Thus, distinction between cDNA and DNA was enabled even with primers located within the same exon, i.e., not intron-spanning. Using samples of cardiomyocytes with photolyzed nuclei, negative re-

sults were obtained, with the exception of a single positive case each by RT-PCR and when reverse transcriptase was omitted.

Laser-assisted cell-picking has previously been used to investigate genomic DNA by PCR, for example to analyze mutations in microscopically identified tumour cells (Becker et al., 1997). Here we demonstrate that it is also a useful tool for mRNA analysis by RT-PCR, and succeeded in identifying a specific mRNA variant (R1-type) from the cholinergic gene locus in a select neuronal population *in situ*. This R1-type mRNA is expressed in the majority of intrinsic cardiac neurons. Since the M-type is predominant in the rat spinal cord (Kengaku et al., 1993), there either exist differences between cardiac and spinal neurons with respect to preferred transcription start, or individual neurons transcribe multiple mRNA variants at different quantities. This study was supported by the DFG, "SFB Cardiopulmonary Vascular System", project C2.

References

- Usdin TB, Eiden LE, Bonner TI et al. *Trends in Neuroscience* 18, 218-224 (1995).
- Misawa H, Takahashi R and Deguchi T. *NeuroReport* 6, 965-968 (1995).
- Kengaku M, Misawa H and Deguchi T. *Mol Brain Res* 18, 71-76 (1993).
- Schütze K and Clement-Sengewald A. *Nature* 368, 667-669 (1994).
- Becker I, Becker KF, Röhl MH. et al. *Histochem Cell Biol* 108, 447-451 (1997).

57

Heat stress, even extreme, does not induce penetration of pyridostigmine into the brain of guinea-pigs

G. Lallement^a, A. Foquin^a, D. Baubichon^a, M.-F. Burckhart^b, P. Carpentier^a, F. Canini^c

^aUnité de Neuropharmacologie, ^bService de Microscopie et d'Imagerie, ^cUnité de Bioénergétique et Environnement, Centre de Recherches du Service de Santé des Armées, BP 87, 38702 La Tronche cedex, France

Stress situation due to forced swim test was recently shown to allow penetration of pyridostigmine (PYR) into the brain of mice. Accordingly, it was suggested that, in troops exposed to emotional stress under conditions of war, as during the Gulf War, the BBB may have unexpectedly become permeable to PYR thus leading to an increased frequency of CNS symptoms. In this study, the entry of PYR into the brain was investigated in guinea-pigs subjected to different heat stress levels. In a first group, guinea-pigs were maintained at room temperature for 2 h, their core temperature remaining stable at about 39.8 °C. In a second group, animals were placed in a climatic chamber in order to keep their core temperature at 41.5 °C for 2 h. In a third group, animals were subjected to a high ambient temperature (42.6 °C) for about 2 h and developed heatstroke symptoms, their core temperature progressively increasing and reaching around 44.3 °C. In each group, the stress of the animals was assessed by measuring the increase of plasma cortisol level and PYR (0.2 mg/kg, s.c.) was injected 90 min after be-

ginning of the experiment. Penetration of the drug into the brain was examined by measurement of acetylcholinesterase (AChE) activity in the cortex, the striatum and the hippocampus of the animals after 30 min. A passage of this drug into the brain was also evaluated autoradiographically after i.v. injection of tritiated PYR 90 min after beginning of the experiment (100 µCi/animal). Whatever the group examined, no entry of PYR into the CNS could be detected. Exposure to an ambient temperature at 42.6 °C for 2 h resulted by itself in a partial inhibition of cerebral AChE activity. Our results, which agree with previous data obtained in humans exposed to heat stress, are opposite to the recent research showing a central passage of PYR in mice following a forced swim stress test. This demonstrated that the penetration of PYR into the brain of rodents under stress depends on the experimental conditions used (animal species, nature of the stressor, etc.). Extrapolations to man, of results primarily obtained in rodents about central passage of a drug under stress, must thus be done very carefully.

58 Cytosolic phospholipase A2 (cPLA2) distribution in murine brain and functional studies indicate that cPLA2 does not participate in muscarinic receptor-mediated signaling in neurons

T.R. Lamar^a, L.L. Lautens^b, X.G. Chiou^a, J.D. Sharp^a, W.S. Young III^c,
D.L. Sprague^b, L.S. Ross^b, C.C. Felder^a

^aNeuroscience Discovery, Lilly Research Labs, Indianapolis, Indiana 46285; ^bDept. of Biological Sciences, Ohio University, Athens, OH 45701;
^cLCMR, National Institute of Mental Health, Bethesda, MD 20892, USA

Muscarinic receptors couple to a variety of signal transduction pathways including the activation of phospholipase A2 (PLA2) and subsequent release of arachidonic acid (reviewed in 1). The m2 and m4 receptor subtypes augment a previously stimulated arachidonic acid release while the m1, m3, and m5 receptors stimulate arachidonic acid release more directly. Arachidonic acid and some of its eicosanoid metabolites regulate neural function through modulation of ion channels, second messenger systems, gene expression, and neurotransmitter uptake and release. Relatively little is known about which PLA2 is involved in muscarinic receptor signaling or the physiologic consequences of the eicosanoid second messengers generated following muscarinic receptor stimulation.

The family of PLA2 enzymes are structurally diverse, yet all catalyze the hydrolysis of the acyl bond at the sn-2 position of membrane phospholipids to release arachidonic acid and lyso-lipids (reviewed in 2). These products are precursors of bioactive eicosanoids and platelet activating factor. Three distinct classes of PLA2 have been well characterized: a) the 14-kDa secreted PLA2 enzymes; b) the 80-kDa calcium-independent cytosolic PLA2 (iPLA2); and c) the 85-kDa calcium-dependent cytosolic PLA2 (cPLA2). The 85-kDa cPLA2 has been considered a potential effector in G protein-coupled receptor signaling for several reasons. cPLA2 is activated and translocates to cell membranes at submicromolar calcium concentrations achieved in the cytoplasm after stimulation of calcium mobilizing plasma membrane receptors. cPLA2 phosphorylation and presumably activation has been demonstrated for several ligands including ATP, thrombin, and bombesin. cPLA2 exhibits strong preference for deacylation of arachidonic acid over other fatty acids. These properties make cPLA2 a promising candidate as an effector involved in receptor-dependent signal transduction and neurotransmission. While ample evidence supports the role of cPLA2 in inflammatory processes, such direct evidence is lacking for the involvement cPLA2 in neurotransmitter function.

Muscarinic receptors couple to the release of arachidonic acid in several cell types including neurons (3) and fibroblasts (4), presumably through activation of an uncharacterized calcium-dependent phospholipase A2. The 85-kDa cPLA2 was recently implicated as an effector in muscarinic receptor-stimulated arachidonic acid release in 1321N1 astrocytoma cells (5). Therefore, we investigated the potential role of cPLA2 in muscarinic receptor-mediated signal transduction through expression studies in three different cell lines. U-373 MG cells were selected because they lacked cPLA2 and CHO and COS-1 cells because they express cPLA2 naturally at low levels therefore providing reasona-

ble hosts for experiments designed to augment the endogenous levels of protein. PLA2 activity in extracts from CHO, U373, and COS cells transfected with cPLA2 cDNA were significantly increased over untransfected control cells. However, in none of the cell lines did the co-expression of muscarinic receptor and cPLA2 result in a significant increase in muscarinic receptor-mediated arachidonic acid release over cells expressing muscarinic receptor alone. These data suggest that muscarinic receptors do not couple through cPLA2 to release arachidonic acid.

Muscarinic receptor-dependent release of AA was previously shown in neurons in primary culture from various brain regions including spinal cord and hippocampus (3). The distribution of cPLA2 mRNA and cPLA2 immunoreactivity in murine brain were determined in order to investigate a potential role for cPLA2 in neurotransmission. cPLA2 mRNA was expressed in white matter, including cells contained within linear arrays characteristic of interfascicular oligodendrocytes. cPLA2 immunoreactivity in white matter was evident throughout the processes of fibrous astrocytes. cPLA2 expression in gray matter was confined to astrocytes at the pial surface of the brain. cPLA2 mRNA was detected in pia mater, both at the brain surface and inner core of the choroid plexus. The prevalence of cPLA2 expression in regions adjacent to fluid-containing compartments or vasculature suggests that cPLA2 may be involved in transport processes or in the maintenance of permeability barriers within the central nervous system. cPLA2 may not be directly linked to neurotransmission since cPLA2 mRNA and cPLA2 immunoreactivity were undetectable in neurons of murine brain. Support or regulation of neurotransmission may be provided through the activity of cPLA2 in glial cells.

- 1 Felder C.C. Muscarinic acetylcholine receptors: signal transduction through multiple effectors. *FASEB J* 9:619-625 (1995).
- 2 Dennis E.A. The growing phospholipase A2 superfamily of signal transduction enzymes. *Trends in Biochemical Sciences*. 22:1-2 (1997).
- 3 Kanterman R.Y., Ma A.L., Briley E.M., Axelrod J., Felder C.C. Muscarinic receptors mediate the release of arachidonic acid from spinal cord and hippocampal neurons in primary culture. *Neurosci Lett* 118:235-237 (1990).
- 4 Felder C.C., Dieter P., Kinsella J., Tamura K., Kanterman R.Y. and Axelrod J.A. transfected m5 muscarinic acetylcholine receptor stimulates phospholipase A2 by inducing both calcium influx and activation of protein kinase C. *J Pharmacol Exp Therap* 255:1140-1147 (1990).
- 5 Bayon Y., Hernandez M., Alonso A., Nunez L., Garcia-Sanchez J., Leslie C., Sanchez Crespo M. and Nieto M.L. Cytosolic phospholipase A2 is coupled to muscarinic receptors in the human astrocytoma cell line 1321N1: characterization of the transducing mechanism. *Biochemical Journal*. 323:281-287 (1997).

59 Multiparametric analysis of the trigger of soman induced epileptic seizures in rats. Correlation between enzymatic, neurochemical and electrophysiologic data

L. Tonduli, G. Testylier, M.I. Pernot, G. Lallement

CRSSA, Neuropharmacology Laboratory, La Tronche, France

Soman, an anticholinesterasic neurotoxic drug, induced epileptic seizure during severe intoxications. The neuropathological lesions are linked to the appearance of the seizures. But their trigger conditions remain still unknown and a great variability between animals is observed.

We have developed in the laboratory a new microdialysis technique allowing an 'in vivo' determination of cortical acetylcholinesterase activity in free moving rats. On the same rat, we have associated this measure with the determination of extracellular acetylcholine concentration by microdialysis and to the EEG recording and analysis.

We have follow these three sets of neurophysiological data during soman intoxication. We have measured in vivo the cortical acetylcholinesterase (AChE), we have followed in the cortex acetylcholine (ACh) level and we have recorded electro encephalographic (EEG) signal and analysed its power spectrum to extract energies especially in the gamma bands (30–40 Hz). We have correlated these data to distinguish three populations of rats, rats

with no seizures and no spiking activity, rats with only spiking activity and rats with seizures.

We have determined the threshold where epileptic seizures occur after injection of soman. AChE inhibition must be over 70%, ACh extracellular level must be over 200-fold the baseline level. However, even with AChE inhibition over 70% and ACh increase over 200-fold, the animals which present an increase in gamma band over three-fold the baseline level did not start epileptic seizure. In the same conditions animals with no increase of gamma band started epileptic seizures with a delay from 10 to 49 min after soman injection.

This method offers a new way to develop medication against poisoning with anticholinesterasic neurotoxics. We have shown that the AChE inhibition and ACh release are necessary but not sufficient parameters to predict the appearance of epileptic seizure. After intoxication, a more integrated behaviour of the rat, like the response in gamma band, will at least decide if the animal will exhibit or not epileptic fit.

60 Cholinesterase (ChE) activity in normotensive rat (WKY) and spontaneous hypertensive rat (SHR)

B. Leconte^a, C. Labat^b, L. Walch^a, J.P. Gascard^a, C. Brink^a, X. Norel^a

^aCentre Chirurgical Marie Lannelongue, CNRS ERS 566, 133, av. de la Résistance, 92350 Le Plessis Robinson;

^bINSERM U337, 15, rue de l'École de Médecine, 75006 Paris, France

Intravenous (i.v.) or intracerebroventricular injection of cholinergic agents such as acetylcholine (ACh) and physostigmine (ChE inhibitor), can modify blood pressure in the rat (Krstic and Djurkovic, 1978; Brezenoff and Rusin, 1974). The injection i.v. of physostigmine induces a greater increase of the mean arterial pressure (MAP) in SHR when compared with WKY (Buccafusco and Spector, 1980). These results could be explained by different ChE and/or choline acetyltransferase (ChAT) activities. Actually, in the brain, specifically in the locus coeruleus, ACh concentration and the activity of ChAT, responsible for ACh synthesis, are increased in the SHR when compared with the WKY (Helke et al., 1980). Furthermore, in the SHR, the densities of the muscarinic receptors are markedly increased in the posterior hypothalamus (Herschkowitz et al., 1983) and decreased in the lung (De Michele et al., 1991). These results suggest that ACh may be involved in hypertension. The ACh regulation of blood pressure may also be due to the hydrolysis of this neurotransmitter by ChE, namely, acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8). The aim of this study was to determine AChE and BChE activities in plasma, erythrocytes, cerebral hemispheres, liver and lung preparations derived from WKY and SHR.

Male WKY and SHR (20 weeks old) were anaesthetised with pentobarbital sodium (60 mg/kg; intraperitoneal injection). The MAP measured with a Gould apparatus was 102 ± 9 mm Hg and 181 ± 6 mm Hg for WKY and SHR, respectively. After this meas-

urement, blood samples were collected in heparinized vials and centrifugated at 3000 rpm/10 min/4 °C. Plasma was collected and erythrocytes were diluted (1/2 in water). Organs (cerebral hemispheres, liver, and lung) were removed. All samples were frozen (–80 °C).

The ChE activities were determined at room temperature using a microplate technique based on Ellman's colorimetric method (Ellman et al., 1961). Plasma and erythrocyte preparations were diluted with phosphate buffer pH 8 (1/54 and 1/540, respectively) whereas organs were pulverised and diluted (1/10) with phosphate buffer, pH 7.4. Tissue preparations were then homogenised by a polytron (400 W) at speed setting 7 (7 times 10 s). The lung and liver homogenates were diluted (1/54) for ChE activity measurement while cerebral hemisphere homogenates were diluted (AChE measurement: 1/162; BChE measurement: 1/54) with phosphate buffer, pH 8. To selectively inhibit either AChE or BChE activities, BW284c51 (1 µM) or iso-OMPA (10 µM) were used, respectively (Austin and Berry, 1953). Maximal velocity (V_{max}) of ChE was measured for plasma and erythrocyte preparations with different concentrations of acetylthiocholine iodide (ACTI) whereas in the tissue preparations, ChE activities were measured in presence of a fixed concentration of ACTI (1 mM) (table I).

A decrease of AChE activity (21% of total ChE activity calculated in WKY blood) was observed in blood derived from SHR. This decrease is principally due to a variation in erythrocyte AChE

Table I. ChE activities in WKY and SHR.

Samples	AChE activity		BChE activity	
	WKY	SHR	WKY	SHR
Plasma	132 ± 5	121 ± 2*	32 ± 1	54 ± 4*
Erythrocyte	1339 ± 127	1040 ± 74*	ND	ND
Liver	268 ± 17	233 ± 14	116 ± 4	126 ± 11
Lung	191 ± 22	293 ± 23*	304 ± 16	361 ± 12*
Cerebral hemisphere	3142 ± 76	2485 ± 66*	217 ± 13	239 ± 27

ChE activities are expressed as, U/L of plasma, U/L of erythrocyte, or mU/g of tissue. ND, not detected. *Data significantly different ($P < 0.05$) from corresponding values obtained in WKY. Values are means ± S.E.M., number of WKY used (6) and number of SHR used (4–15).

activity. An increase of blood BChE activity (1% of total ChE activity calculated in WKY blood) was observed in SHR.

AChE and BChE activities were increased (53% and 19%, respectively) in SHR lung when compared with WKY lung. In SHR cerebral hemispheres, a decrease of AChE activity (21%) was observed. No difference was determined in BChE and ChE activities in cerebral hemispheres and liver, respectively, when SHR and WKY were compared.

This study shows differences in ChE activities, in blood and lung, between SHR and WKY. In addition, the different brain cholinergic regulation of the systemic blood pressure, between SHR and WKY, may involve the AChE activity in cerebral hemispheres.

References

- Austin L, Berry WK (1953) Two selective inhibitors of cholinesterase. *Biochem. J.* 54, 695–700
- Brezenoff HE, Rusin J (1974) Brain acetylcholine mediates the hypertensive response to physostigmine in the rat. *Eur. J. Pharmacol.* 29, 262–266
- Buccafusco JJ, Spector S (1980) Role of central cholinergic neurons in experimental hypertension. *J. Cardiovasc. Pharm.* 2, 347–355
- De Michele M, Cavallotti C, Amenta F (1991) Autoradiographic localization of muscarinic acetylcholine receptors in the rat pulmonary vascular tree. *Eur. J. Pharmacol.* 192, 71–78
- Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7, 88–95
- Helke CJ, Muth EA, Jacobowitz DM (1980) Changes in central cholinergic neurons in the spontaneously hypertensive rat. *Brain Res.* 188, 425–436
- Hershkowitz M, Eliash S, Cohen S (1983) The muscarinic cholinergic receptors in the posterior hypothalamus of hypertensive and normotensive rats. *Eur. J. Pharmacol.* 86, 229–236
- Krstic MK, Djurkovic D (1978) Cardiovascular response to intracerebroventricular administration of acetylcholine in rats. *Neuropharmacology* 17, 341–347

61 Repetitive and spontaneous acetylcholine release from motor nerve terminals induced by a neurotoxin isolated from *Conus ermineus* venom

F. Le Gall^{a, b}, P. Favreau^{a, b}, E. Benoit^b, Y. Letourneux^a, J. Molgo^b

^aLaboratoire de Synthèse et Etudes de Substances Naturelles à Activités Biologiques, Université de La Rochelle, Pôle Sciences, avenue Marillac, 17042 La Rochelle cedex;

^bLaboratoire de Neurobiologie Cellulaire et Moléculaire, UPR-CNRS 9040, bât. 32, 1, avenue de la Terrasse, 91198 Gif-sur-Yvette cedex, France

Marine snails of the genus *Conus* are venomous animals classified according to their feeding behaviour into piscivorous, molluscivorous and vermivorous species (Kohn, 1983). Several peptide toxins have been isolated from the venom of fish-hunting cone snails: α -conotoxins active on nicotinic receptors (Myers and Cruz, 1993); μ -conotoxins which block voltage-dependant Na^+ channel in skeletal muscle (Cruz and Gray, 1986) and ω -conotoxins which interact with voltage-gated Ca^{2+} channels subtypes (Miljanich and Ramachandran, 1995). Each of these toxins potentially block neuromuscular transmission in vertebrates producing flaccid muscle paralysis. Because of their specific actions on key elements of excitable cells and receptors, conotoxins have been widely used as tools for studying synaptic transmission mechanisms and for characterizing ion channels and receptor subtypes (for a recent review, see Olivera, 1997).

The observation that the venom of the fish-hunting cone snail *C. ermineus* elicits prominent muscle contraction in fish prompted us to purify the toxin(s) responsible for the observed action

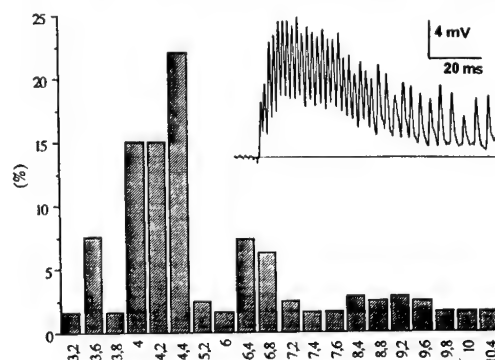


Figure 1. Interval distribution of repetitive EPPs. Recording made intracellularly at the junction fibers treated with $\delta\text{A-EVIA}$ (200 nM) showed repetitive EPPs following a single nerve stimulus. The firing rate was initially at about 300 Hz and slowed with time (see insert). Measured intervals between the EPPs were averaged for 6 stimuli on the same fiber and plotted in the histogram.

of the venom and to characterize its mode of action on excitable membranes and neurotransmitter release from nerve terminals.

The present communication reports that *C. ermineus* venom contains a neuropeptide named δ A-EVIA (M_r , 3300 D) which selectively increases synaptic efficacy at the vertebrate neuromuscular junction by increasing the number of synaptic responses evoked by a single nerve stimulus as well as the spontaneous quantal acetylcholine release from motor nerve terminals through a sodium dependent mechanism.

δ A-EVIA, when applied to isolated frog nerve-muscle preparations (cutaneous pectoris) bathed in normal Ringer's solution causes a long-lasting spontaneous contraction of the muscle fibers which prevented stable intracellular recordings. In order to avoid muscle movements, excitation-contraction was uncoupled by formamide treatment (see del Castillo and Escalona de Motta, 1978). Intracellular recordings from formamide-treated junctions revealed that δ A-EVIA (200 nM) induced repetitive action potentials in response to a single nerve stimulus. These action potentials were triggered by spontaneous and repetitive endplate potentials (EPPs) which could attain high frequencies (figure 1). This repetitive synaptic activity had variable duration, could last tens of seconds and was not or little modified by increasing Mg^{2+} concentration in the extracellular medium or by addition of (+)-tubocurarine (3 μ M).

Analysis of the quantal contents of EPPs, in preparations equilibrated with low Ca^{2+} (0.3 mM) and high Mg^{2+} (6 mM) Ringer's solution revealed that δ A-EVIA (200 nM) did not modify the average number of quanta released during the first phasic EPP. Thus, it appears that the main effect of δ A-EVIA is to alter the 1:1 input-output ratio of the neuromuscular junction. In other words, δ A-EVIA induces a marked repetitive synchronous release of acetylcholine in response to a single nerve stimulus. Interes-

tingly, the repetitive EPPs in a given train exhibited facilitation that could last tens of seconds.

In addition, δ A-EVIA (200 nM) after a delay of about 40 min increased spontaneous quantal acetylcholine release, recorded as miniature endplate potential (MEPP) frequency, in a nominally Ca^{2+} free medium supplemented with EGTA (2 mM). Such an enhancement of MEPP frequency could be either prevented or reversed by the sodium channel blocker, tetrodotoxin (1 μ M). These results indicate that the increase of MEPP frequency caused by δ A-EVIA is related to sodium entry into motor nerve endings.

In conclusion, δ A-EVIA isolated from *C. ermineus* venom constitutes a new excitotoxin that interacts with voltage-gated Na^+ channels modifying nerve terminal excitability, neurotransmitter release and synaptic efficacy at the vertebrate neuromuscular junction.

References

- Cruz L.J., Gray W.R., Olivera B.M., Zeikus R.D., Kerr L., Yoshikami D., Moczydlowski E (1985) *Conus geographus* toxins that discriminate between neuronal and muscle sodium channels. *J Biol Chem* 260, 9280-9288
- Del Castillo J., Escalona de Motta G (1978) A new method for excitation-contraction uncoupling in frog skeletal muscle. *J Cell Biol* 78, 782-784
- Kohn A.J. (1983) Feeding biology of gastropods *The mollusca* 5, 1-63
- Miljanich G.P., Ramachandran J (1995) Antagonists of neuronal calcium channels: structure, function, and therapeutic implications. *Annu Rev Pharmacol Toxicol* 35, 707-734
- Myers R.A., Cruz L.J., Rivier J., Olivera B.M. (1993) *Conus* peptides as chemical probes for receptors and ion channels. *Chem Rev* 93, 1923-1936
- Olivera B.M. (1997) *Conus* venom peptides, receptor and ion channel targets, and drug design: 50 million years of neuropharmacology. *Mol Biol Cell* 8, 2101-2109

Developmental expression and localisation of acetylcholinesterase in rat muscles

C. Legay, J. Massoulié

Laboratoire de Neurobiologie, CNRS-URA 1857, École Normale Supérieure, 46, rue d'Ulm, 75005 Paris, France

The localisation of acetylcholinesterase (AChE) is a key factor in the control of cholinergic transmission. It is determined by the AChE forms expressed by a cell: AChE exists under a multiplicity of forms generated by alternative splicing of a unique pre-mRNA (Massoulié et al., 1993). Two main types of subunits, T and H, contain the same catalytic domain but differ by their C-terminus. H subunits are glycosylated and therefore anchored in cell membranes. T subunits may be associated with structural subunits such as a 20 kDa protein (G_4^b) or a specific collagen (ColQ) that anchor AChE respectively in membranes and in the basal lamina.

They may also be secreted as oligomeric forms or tethered by the T peptide in membranes. The regulation of alternative splicing and the availability of the associated proteins in a specific tissue determine the cellular targeting of the enzyme.

T mRNAs are the only mRNAs detected in adult brain and muscle whereas H mRNAs are the main transcripts detected in hematopoietic cells, together with variable amounts of T and R mRNAs. In R transcripts, the intron separating the last 3' constitutive exon from the H alternative exon is retained. This last trans-

cript contains an in-frame stop codon and is at least partly polyadenylated. Its presence is generally associated with that of the H transcript. So far the protein has not been demonstrated in vivo although secreted monomeric AChE that could correspond to this form has been observed (Navaratnan et al., 1991). It remains that the R sequence could also be a regulator of splicing choices or simply leakage of splicing. At early stages of embryonic development (E13-E14), T, R and H transcripts are expressed in this decreasing quantitative order probably reflecting splicing choices in myoblasts and early differentiating myotubes. In fact, C2C12 myoblasts express H and T mRNAs whereas myotubes express mainly T transcripts together with a small proportion of R transcripts. The onset of innervation and the maturation of the synapse coincide with the progressive and exclusive expression of T mRNAs (Legay et al., 1995). However, this splicing choice could be dictated by the differentiation of the myotubes rather than by the presence of the nerve. C2C12 myotubes transiently transfected with a mini-gene containing the 3' alternative introns and exons produce only T subunits (Legay et al., in preparation).

The muscle synthesizes a collagen protein ColQ that interacts with the T peptide of AChE through its N-terminus and anchors the enzyme in the extracellular matrix (Krejci et al., 1997; Bon et al., 1997). Like the subunits of the acetylcholine receptor and other components associated with the cholinergic transmission, accumulation of both proteins are observed in slow and fast muscles at the neuromuscular junctions. Accumulation of the specific mRNAs is partly responsible for this phenomenon. T mRNAs are accumulated in all muscles under the synaptic contacts as soon as E14 (Legay et al., 1995) but although the ColQ gene is transcribed at that stage, ColQ mRNAs were not detected at specific zones. Synaptic accumulation of ColQ mRNAs could be visualized by this at a late post-natal stage of differentiation in the sternomastoid muscle, a fast muscle but not in slow muscles. These results suggest that the localisation of ColQ mRNAs depends on the pattern of muscle activity. RNase protection assays corroborate these results in adult muscles showing that the level of ColQ mRNAs is equivalent in junctional and extrajunctional domains of soleus muscle whereas these mRNAs are only present in junctional areas of the sternomastoid muscle. However, in the soleus muscle, an increased density of the ColQ protein is detectable at the neuromuscular junction by immunohistochemistry. This suggests that, in this muscle, additional mechanisms ensure targeting

and/or anchoring of ColQ. For example, if the ColQ protein associate with a target in the synaptic cleft, the localisation of ColQ would be linked to the distribution of the target, itself being subjected to the state of development and differentiation of the cell.

References

- Bon S., Massoulié, J. (1997) Quaternary associations of acetylcholinesterase. I. Oligomeric associations of T subunits with and without the amino-terminal domain of the collagen tail. *J. Biol. Chem.* 272, 3007-30015
- Krejci E., Thomine S., Boschetti N., Legay C., Sketelj J., Massoulié J. (1997) The mammalian gene of acetylcholinesterase-associated collagen. *J. Biol. Chem.*, 272, 22840-22847.
- Legay C., Huchet M., Massoulié J., Changeux J.P. (1995) Developmental regulation of acetylcholinesterase transcripts in the mouse diaphragm: alternative splicing and localisation. *Eur. J. Neurosci.* 7, 1803-1809.
- Massoulié J., Pezzementi L., Bon S., Krejci E., Vallette F. (1993) Molecular and cellular biology of cholinesterases. *Prog. Neurosci.*, 41, 31-91.
- Navaratnam B.S., Priddle J.D., McDonald B., Esiri M.M., Robinson J.R., Smith A.D. (1991) Anomalous molecular form of acetylcholinesterase in cerebrospinal fluid in histologically diagnosed Alzheimer's disease. *Lancet* 337, 447-450.

63

Predicted secondary structure of a nicotinic acetylcholine receptor subunit. Incorporation of predicted solvent accessibility and experimental data into a two dimensional representation

N. Le Novère, P.J. Corringer, J.P. Changeux

Neurobiologie Moléculaire, Institut Pasteur, 75724 Paris cedex, France

A refined prediction of the nicotinic acetylcholine receptor (nAChR) subunits' secondary structure was computed with third generation algorithms. The four selected programs, PHD, PREDATOR, DSC, NNSSP, based on different prediction approaches, were applied to each sequence of an alignment of nAChR and 5-HT₃ receptor subunits, as well as of a larger alignment with additional related subunits sequences from glycine and GABA receptors. A consensus prediction was computed through a 'winner takes all' method. By integrating the probabilities obtained with PHD, DSC and NNSSP, this prediction was filtered in order to eliminate the singletons and to establish more precisely the structure limits (only 4% of residues were modified in this way). The final consensus secondary structure includes nine α -helices (24.2% of the residues, average length = 13.9) and 17 β -strands (22.5% of the residues, average length = 6.6). The large extracellular domain is predicted to be mainly composed of β -strands, with only two helices at the amino-terminal end. The transmembrane segments are predicted to be in a mixed α/β topology (with a predominance of α -helices), with no known equivalent in the current protein database. For the cytoplasmic domain only two amphipathic helices are predicted at both ends, with no periodic

structures predicted for the remaining part (of variable length). The structured segments correspond to the more conserved regions, as defined by an analysis of sequence conservation per position performed on 152 superfamily members.

The solvent accessibility of each residue was also predicted from the multiple alignments with PHDacc. Each segment with more than three residues predicted exposed was considered to be external to the core protein. These data, along with a disulphide bond and a segment localised by electron diffraction, composed an envelop of structural constraints allowing to propose a partial folding of a nAChR subunit. Each subunit of the nAChR superfamily, polarised, possesses two different binding faces, oriented toward two other subunits within the oligomer. A 2D representation of the secondary structure is proposed which is fully compatible with experimentally identified residues known to belong to these two faces. In this representation, the segments of variable length are placed outside the core protein. This representation is not a tertiary structure and does not lead to prediction of any β -interaction. However, it provides a basic framework for further mutagenesis investigations, and for fold recognition (threading) searches.

64 Effect of continuous administration of pyridostigmine on the activity of functional acetylcholinesterase in guinea-pig muscle and brain.

M.C. Lintern^a, J. Wetherell^b, M.E. Smith^a

^aDepartment of Physiology, The Medical School, University of Birmingham, Birmingham, B15 2TT;

^bBiomedical Sciences Dept., CBD, Porton Down, Salisbury, Wiltshire, SP4 0JQ, UK

We have shown previously that repeated treatment in mice with the reversible anticholinesterase pyridostigmine caused increases in acetylcholinesterase (AChE) activity in skeletal muscle after the initial inhibition had worn off. Furthermore, pretreatment with pyridostigmine sensitised the muscle to a subsequent dose of the drug administered after the enzyme activity had returned to normal (Lintern et al. 1997a, b). In the present study the effect of continuous administration of pyridostigmine on the activity of AChE in muscle and brain, was studied in the guinea-pig. The drug was administered at a rate of 5.1 µg/h via an implanted osmotic pump. The pump was left in place for 6 days. Control animals received saline, administered in the same way. One group of animals was killed by cervical dislocation at 6 days, with the pump still in place, and other groups were killed at 4 h, 24 h, or 3, 7 or 14 days after removal of the pump. The activity of AChE was determined in the diaphragm and soleus muscles, and in the striatum and cerebellum.

The tissues were homogenised in high salt solution and the AChE molecular forms separated on a 5–20% sucrose density gradient (Haynes et al., 1984). The enzyme activity was determined by the method of Ellman (1961) modified for use with a plate reader. Major peaks of activity representing the G1, G4 and A12 forms were separated in muscle and major peaks representing the G1 and G4 forms were separated in the brain areas. The activity of each molecular form was determined by summing the activities of the fractions contributing to each peak.

Pyridostigmine administration for 6 days inhibited red cell AChE activity by 43 ± 2 (S.E.M.)% where $n = 24$. Figure 1 shows the results obtained for total and functional AChE activity in animals killed after 6 days of continuous administration of the drug (with the pump still in place). In both muscle types the total activity of AChE was significantly higher in the pyridostigmine-treated animals than in the saline treated controls. In general the activity of the predominant (G1, G4, and A12) molecular forms of the enzyme exhibited similar proportional increases. The effect of the drug on the functional A12 activity was particularly marked in the soleus muscles, causing an increase in activity of approximately 80%. After removal of the pump the changes in activity due to the effect of the drug exhibited a variable time course depending on muscle type.

In the striatum there was a slight increase in total, G1 and G4 activity compared to the saline-treated controls, although the changes were not significant. However in the cerebellum the total activity and the G1 and G4 activity was significantly reduced compared to the controls. After removal of the pump the changes in activity due to the effect of the drug exhibited a variable time course depending on the brain area being examined.

The biological half-life of pyridostigmine is approximately 50 min (Maxwell, 1995) but the homogenisation and separation procedures require approximately 24 h for completion. Therefore residual inhibition of the activity by the reversible inhibitor was probably negligible at the time the activity was determined. The decreases in AChE activity in the cerebellum were therefore probably due to down-regulation of the enzyme.

Pyridostigmine does not under normal circumstances cross the blood-brain barrier. However, effects of this drug on brain AChE activity have been reported after specific stress regimes (Friedman et al., 1996). It is not yet clear whether the changes in the enzyme activity reported here are a direct action of the drug, as a consequence of changes in the permeability of the blood brain barrier, or secondary to some peripheral action of the inhibitor. This work has been carried out with the support of CBD Porton Down. (c) British Crown copyright 1998/DERA. Published with permission of the Controller of Her Britannic Majesty's Stationary Office.

References

Ellman GL, Courtney D, Andres V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7, 88–95

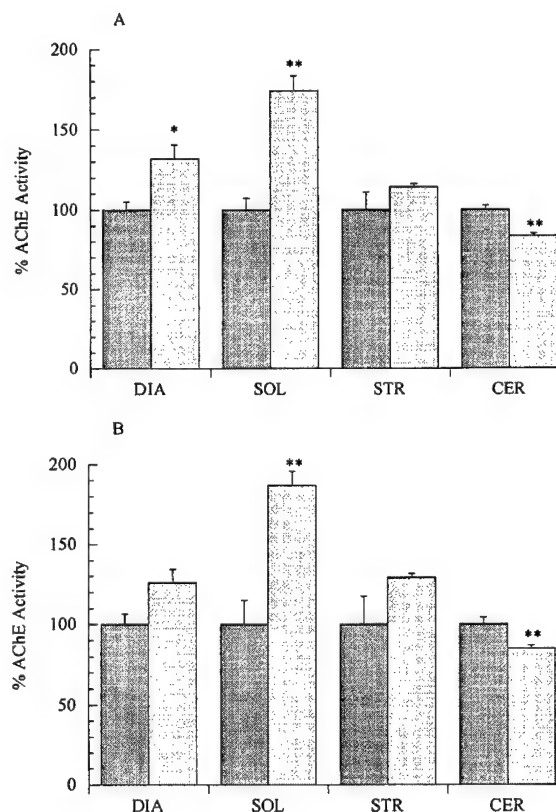


Figure 1. Activity of AChE after 6 days continuous administration of pyridostigmine in diaphragm (DIA) and soleus (SOL) muscles, and striatum (STR) and cerebellum (CER). A. Total activity. B. Functional activity (A12 in muscle and G4 in brain areas). Dark columns, saline-treated; light columns, pyridostigmine-treated. The values are means \pm S.E.M. for four animals in each case. * $P < 0.05$ compared to saline-treated controls (for raw data).

Friedman A, Kaufer D, Shemer J, Handler I, Soreq H, Tur-Kaspa I (1996) Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. *Nature Med* 2, 1382-85

Haynes LW, Smith ME, Smyth DG (1984) Evidence for the neurotrophic regulation of acetylcholinesterase in immature skeletal muscle by β -endorphin. *J Neurochem* 42, 1542-51

Lintern ML, Smith ME, Ferry CB (1997a) Effects of pyridostigmine on acetylcholinesterase in different muscles of the mouse. *Human Exp Toxicol* 16, 18-24

Lintern ML, Smith ME, Ferry CB (1997b) Effect of repeated treatment with pyridostigmine on acetylcholinesterase in mouse muscles. *Human Exp Toxicol* 16, 158-165

Maxwell DM, Brecht KM, Doctor BP, Wolfe AD (1993) Comparison of antidote protection against soman by pyridostigmine, HI-6 and acetylcholinesterase. *J Pharmacol Exp Ther* 264, 1085-9

65

Bone morphogenetic protein-9 modulates acetylcholine content and choline acetyltransferase and vesicular acetylcholine transporter gene expression in the SN56 murine septal cell line

I. Lopez-Coviella^a, B. Berse^a, R.S. Thies^b, J.K. Blusztajn^a

^aDept. of Psychiatry and Dept. of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, MA 02118

^bGenetics Institute Inc., Cambridge, MA 02140, USA

Bone morphogenetic proteins (BMP) belong to the TGF- β family of cytokines that signal through receptor serine kinases. Their functions in the developing and adult nervous system are poorly understood, but include neurotation, apoptosis, neuronal and glial differentiation, cell survival, and possibly regulation of neurotransmitter phenotype (Ebendal et al., 1998; Mehler et al., 1997). We studied the effects of BMP-9 on acetylcholine (ACh) content and on choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VACHT) mRNA levels of SN56 cells.

SN56 cells were grown in DME medium (containing 4.5 g/L of glucose and 110 mg/L sodium pyruvate) supplemented with 10% fetal bovine serum in the presence or absence of human recombinant BMP-9 for varying time periods. One hour prior to cell collection, the cells were incubated at 37 °C in a physiological salt solution containing 50 μ M neostigmine and 5 μ M choline. The cells were washed once to remove extracellular choline and collected in methanol. Water-soluble choline-containing compounds were extracted and ACh content was measured by HPLC coupled to postcolumn enzymatic derivatization and electrochemical detection.

To determine ChAT and VACHT mRNA levels, total RNA from SN56 cells was extracted using the guanidinium thiocyanate-phenol/chloroform method (Chomczynski and Sacchi, 1987). Northern blot analysis of equalized RNA samples was performed with VACHT and ChAT cDNA probes as previously described (Berse et al., 1995).

Incubation of SN56 cells with BMP-9 (10 ng/mL) for varying periods of time caused initial increases in ACh content (by 45% and 60% after 12 and 24 h, respectively). However, by 48 h BMP-9 caused a reduction in the cellular ACh content (by 40%) which remained stable for up to 72 h (figure 1). Consistent with this time-course, in cells treated for 48 h with varying concentrations of BMP-9, ACh content declined by as much as 60% in a concentration-dependent, saturable fashion with an EC₅₀ for BMP-9 of 1.3 ng/mL.

Messenger RNA levels for ChAT and VACHT were reduced by 41% and 50%, respectively, 48 h after treatment with BMP-9. No significant changes were observed in the mRNA levels for these two cholinergic markers following shorter incubation periods with BMP-9 (i.e., at 12 and 24 h).

These data show that initial exposure of SN56 cells to BMP-9 results in increased ACh content, followed by a reduction in the levels of this neurotransmitter. This later reduction may be a con-

sequence of down-regulation of VACHT and ChAT gene expression, since our results also show reduced levels of the mRNAs coding these two proteins. Whether this effect is entirely mediated by a direct BMP-9 signaling pathway through specific SMAD proteins, or results from the concomitant activation of signaling pathways involving other factors, remains to be determined.

These findings provide further evidence that neuronal cells respond to bone morphogenetic proteins, and suggest that BMP-9 can directly or indirectly affect the expression of the cholinergic phenotype. Supported by a National Institute on Aging grant AG09525.

References

Berse B. and Blusztajn J.K. (1995) Coordinated up-regulation of choline acetyltransferase and vesicular acetylcholine transporter gene expression by the retinoic acid receptor α , cAMP, and

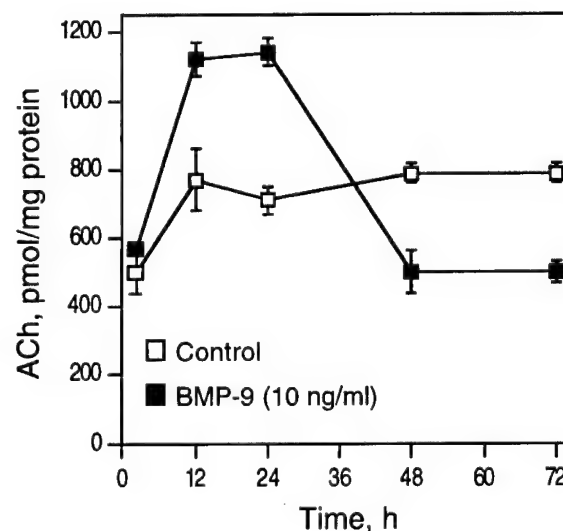


Figure 1. Time-course of the effect of BMP-9 on ACh content in SN56 cells. ACh content was measured by HPLC. Data are reported as means \pm S.E.M.

CNTF/LIF signaling pathways in a murine septal cell line J. Biol. Chem. 270: 22101-22104.

Chomczynski P. and Sacchi N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction Anal. Biochem. 162:156-159, 1987.

Ebendal T., Bengtsson H., and Soderstrom S. (1998) Bone morphogenetic proteins and their receptors: potential functions in the brain. J. Neurosci. Res. 51: 139-146.

Mehler M.F., Mabie P.C., Zhang D., and Kessler J.A. (1997) Hematopoietic and inflammatory cytokines in neural development TINS 20:309-317, 1997.

66

Acceleration of oxime-induced reactivation of acetylcholinesterase-organophosphate conjugate and ^{31}P NMR detection of phosphoryl oxime from the conjugate

C. Luo, Y. Ashani, B.P. Doctor

Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307-5100, USA

The acute toxicity of organophosphorous compounds (OP) is usually attributed to the irreversible inhibition of AChE by these compounds. Reactivation of the OP-inhibited AChE by oximes is the primary reason for their effectiveness in the treatment of OP poisoning. Reactivation by oxime is accelerated by quaternary ligands such as decamethonium and SAD 128, which are devoid of nucleophilicity (1). We have shown that among the three different quaternary ligands examined, only edrophonium and decamethonium can accelerate the oxime-induced reactivation of the enzyme conjugate (2). The peripheral site ligand propidium slowed the reactivation process with all oximes tested. Determination of the kinetic reactivation rate constants with and without the accelerating ligands showed that in the presence of 50 μM edrophonium, 3.3- to 12.0-fold accelerations were obtained with 2-PAM, TMB4 and toxogonin as reactivators, and 1.6- to 3.0-fold enhancements were observed in the presence of 200 μM decamethonium with these oximes. However, the reactivation by HI-6 was not accelerated by either of the two ligands. Examination of edrophonium acceleration of oxime-induced reactivation with high (12.5 nM) and low (1.25 nM) enzyme:OP concentration of the reactivation with 12.5 nM enzyme:OP was significantly lower than that with 1.25 nM enzyme:OP, indicating that acceleration may involve protection of the reactivated enzyme by the added ligand from re-inhibition by the putative product, the phosphoryl oxime (POX).

Acceleration of reactivation of DEPQ [7-(O,O-diethyl-phosphinyloxy)-1-methyl-quinolinium methylsulfate]-inhibited FBS and wild-type mouse AChE by edrophonium was the same with AChEs from these two sources. However, with DEPQ-inhibited AChE, only reactivations by toxogonin and TMB4 were accelerated by edrophonium. Reactivations by HI-6, 2-PAM and MMB4 were not significantly affected in the presence of edrophonium. Comparison of the initial reactivation rate constants and overall reactivation rate constants of DEPQ-inhibited wild-type mouse AChE by these five oximes supported the hypothesis that edrophonium prevents the re-inhibition of the reactivated enzyme by POX. If the POX is produced by reaction of oxime with the enzyme:OP and the re-inhibition of the reactivated enzyme is caused by the POX, the initial reactivation rate should be significantly faster than the overall reactivation rate, since re-inhibition occurs only after the initial stage when a sufficient amount of POX is accumulated in the reactivation medium. The initial reactivation rate constants of DEPQ-inhibited wild-type mouse AChE were 5.4- and 4.2-fold higher than those of the overall rate constants with toxogonin and TMB4. On the other hand, very little difference was observed between the initial and overall rate constants

with the other three oximes. Approximately 2.5-fold increase in the reactivation rate constants with toxogonin and TMB4 was observed in the presence of 10 μM edrophonium. No effect was observed with the other three oximes. When initial and overall reactivation rate constants of wild-type mouse AChE inhibited by nerve agent sarin were compared, it was found that the reductions of the overall rate constants compared to the initial rate constants were greater than those observed with DEPQ-inhibited AChE with most of the oximes. With toxogonin and TMB4, 23.3- and 19.5-fold reductions of the overall rate constant were observed. Approximately 3-fold decreases in the overall rate constants were observed with MMB4 and 2-PAM. The initial and overall rate constants were essentially the same when HI-6 was used. Accelerations by edrophonium were more pronounced for the reactivation of sarin-inhibited AChE than DEPQ-inhibited AChE with most of the oximes. In the presence of 10 μM edrophonium, the overall reactivation rate constants of sarin-inhibited AChE by toxogonin and TMB4 were increased by the greatest amount, 4.7- and 4.3-fold, respectively. The increase was moderate with MMB4 and 2-PAM, 1.8- and 1.4-fold, respectively, and there was no change with HI-6. The results of experiments with sarin indicate that POX re-inhibition was more pronounced for the reactivation of sarin-inhibited AChE than DEPQ-inhibited AChE.

Recently it was shown that aspartate 74, a residue near the entrance of the active center gorge of AChE, is the primary determinant in governing specificity of the enzyme to cationic OP (3). The mutant D74N displayed 2-3 orders of magnitude decrease in the inhibition activity of cationic OPs. POXs are analogues of those cationic OPs and should demonstrate similar properties. Thus for this specific mutant, edrophonium-induced acceleration will not be evident if the acceleration is produced by preventing POX re-inhibition of the reactivated enzyme during reactivation. When mutant D74N was employed, no significant edrophonium-induced acceleration could be observed in reactivation of DEPQ-inhibited AChE by toxogonin and TMB4, and the time course of reactivation fitted to a mono-exponential phase association kinetic model. The data from mutant enzyme substantiated the involvement of edrophonium in protecting POX re-inhibition of reactivated enzyme formed during the reactivation of OP-inhibited AChE.

It was recently also shown that OP hydrolase can effectively hydrolyze quaternary ammonium-containing OPs (4). It is possible that POXs might be also hydrolyzed by it since they share common structural features. If the putative POXs formed during reactivation are hydrolyzed by OPH, reactivation of OP-AChE

conjugate may be accelerated by the presence of OP hydrolase. Parallel studies with edrophonium and rabbit serum OP hydrolase showed this to be the case. Reactivation of DEPQ- or sarin-inhibited AChE was accelerated in the presence of either edrophonium or OPH.

POXs are putative products during the reactivation of certain OP-AChE conjugates by oxime reactivators. No direct evidence had been obtained for the existence of POXs in the reactivation media, but they were indicated by kinetic studies to be present in some cases (5, 6). Several chemically synthesized POXs have been tested recently and shown to have a greater ability to inhibit AChE or BChE compared with the parent OPs (7). Therefore, isolation and identification of the POXs from the reactivation media is of importance. ^{31}P NMR measurements of the mixture of MEPQ and oximes showed that the POX between MEPQ and HI-6 may not exist for any significant length of time due to the extreme unstability of this POX in solution. However, POXs between MEPQ and toxogonin or TMB4 were found to be stable enough for ^{31}P NMR detection at pH 4.0. To isolate POX directly from the reactivation media of MEPQ-inhibited AChE, approximately 0.2 μmol of FBS AChE was absorbed to a small procainamide Sepharose 4B affinity gel-column to create an enzyme conjugate-reactivated enzyme cycling situation. Reactivation media contained 0.5 mM reversible inhibitor, edrophonium, and 1 mM oxime to keep the newly-formed POX from inhibiting the reactivated enzyme. After concentrating the reactivation media 100-fold, POXs formed during reactivation of MEPQ-inhibited AChE by toxogonin and TMB4, but not by HI-6, were confirmed for the first time by ^{31}P NMR.

In conclusion, results from this study using different AChEs and a variety of OP and oxime compounds confirmed that acceleration of oxime-induced reactivation of OP-inhibited AChE by some quaternary ligands, such as edrophonium and decamethonium, results from the prevention of POX re-inhibition of reactivated enzyme. OP hydrolase can hydrolyze the POX and also accelerate oxime-induced reactivation. POX re-inhibition during reactivation largely depends on the structure of the oximes and the OPs used. With some oximes like HI-6 and its analogues, POX re-inhibition will not occur either with phosphorylated AChE

(DEPQ-inhibited AChE) or with phosphorylated AChE (MEPQ-, and sarin-inhibited AChE) due to the extremely poor stability of the POXs in solution. With 2-PAM, POX re-inhibition is found only with phosphorylated AChE. POXs formed by toxogonin and TMB4 with phosphorylated AChE are the most stable and can be isolated from the reactivation media. POX re-inhibitions are the most pronounced with toxogonin and TMB4 as reactivators during the reactivation of both the phosphorylated and phosphonylated AChE. Caution should be exercised in using toxogonin and TMB4 for treatment of poisoning with large dose of OP pesticide, since these two oximes may form POXs by direct reaction with free OP in vivo and produce more severe inhibition of AChE than the parent OP.

References

1. Harris LW, Heyl WC, Stitche DL and Broomfield CA (1978) Effects of 1,1'-oxydi-methylene bis-(4-tert-butylpyridinium chloride) (SAD-128) and decamethonium on reactivation of soman- and sarin-inhibited cholinesterase by oximes. *Biochem Pharmacol* 27: 757-761.
2. Luo C, Ashani Y, and Doctor B. P. Acceleration of oxime-induced reactivation of organophosphate-inhibited fetal bovine serum acetylcholinesterase by mono-quaternary and bisquaternary ligands. *Mol Pharmacol* 53:718-726.
3. Hosea NA, Radic Z, Tsigelny I, Berman HA, Quinn DM and Taylor P (1996) Aspartate 74 as a primary determinant in acetylcholinesterase governing specificity to cationic organophosphonates. *Biochemistry* 35:10955-11004.
4. Ashani Y, Leader H, Rothschild N and Dosoretz C (1998) Combined effect of organophosphorous hydrolase and oxime on the rate of reactivation of diethylphosphoryl-acetylcholinesterase conjugates. *Biochem Pharmacol* 55: 159-168.
5. Schoene K (1972) Reaktivierung von O,O-diäthylphosphoryl-acetylcholinesterase: Reaktivierungsrephosphorylierungsgleichgewicht. *Biochem Pharmacol* 21: 163-170
6. Harvey B, Scott RP, Sellers DJ and Watts P (1986) In vitro studies on the reactivation by oximes of phosphorylated acetylcholinesterase: I. On the reactions of P2S with various organophosphates and the properties of the resultant phosphorylated oximes. *Biochem Pharmacol* 35: 737-744.
7. Ashani Y. Personal communication.

67

Complementary binding studies between α -neurotoxin and the nicotinic acetylcholine receptor

S. Malany, E. Ackermann, H. Osaka, P. Taylor

Department of Pharmacology, University of California San Diego, La Jolla, California 92093, USA

The nicotinic acetylcholine receptor (nAChR), which is composed of four homologous subunits with composition $\alpha 2\beta\delta\gamma$, binds agonist and competitive antagonists at the boundary between the $\alpha\gamma$ and $\alpha\delta$ interfaces. Recently Tsigelny et al.¹ have developed and refined a sequence homology model of the extracellular domains of individual subunits in nAChR. This model highlights three distinct regions of sequence in the α -subunit and four regions in the δ and γ subunits on the face opposing the α -subunit that contribute to ligand binding and specificity.

Conserved determinants in domain III of the α -subunit that influence affinity for α -toxin have previously been identified in our laboratory². Binding sites between snake venom toxin *Naja mossaambica mossaambica* (Nmml), expressed from recombinant

DNA bacterial systems, and mouse muscle nAChR, expressed transiently from transfected cDNAs on the surface of human embryonic kidney cells, were determined by utilizing site-directed mutagenesis and measuring equilibrium dissociation constants (K_d values) by competition against initial rates of [^{125}I]- α -bungarotoxin³. The binding assays were conducted in the presence and absence of α -conotoxin M1, which shows significant site-selectivity (104 difference in K_d)⁴, for the $\alpha\delta$ binding site over the $\alpha\gamma$ site. These α -subunit determinants include: 1) a conserved tyrosine (Y190) believed to contribute to stabilization of positive charges on the toxin; 2) a negative charge (D200) believed to affect binding at the $\alpha\gamma$ interface but not at the $\alpha\delta$ interface; and 3) residues (V188) and (P197), which when mutated to charged re-

sides large decreases in affinity result presumably because of charge repulsion. The resulting decreases in K_d values varied from no change to 40-fold at the $\alpha\delta$ site and 20- to 500-fold at the $\alpha\gamma$ site.

The three fingered α -neurotoxin Nmml shares ~60% residue identity with erabutoxin a for which the X-ray crystal structure has been solved⁵. Modification of cationic side chains K27E and R33E on loop II and K47A on loop III resulted in 1 to 2 orders of magnitude shift at the $\alpha\delta$ -site and 1 to 4 orders of magnitude shift at the $\alpha\gamma$ -site². Analysis of these combinations of mutations in both the receptor and the α -toxin suggest that key cationic side chains on the toxin bind in close proximity to this segment encompassing residues 180–200 of the receptor⁶. In addition, these studies have confirmed the ligand affinity distinctions between the $\alpha\delta$ and $\alpha\gamma$ sites and have also suggested that the α -toxin interacts at a multipoint attachment with both α -subunit and δ/γ -subunit determinants.

This presentation will concentrate on results from continued studies involving the correlation of kinetic data for combinations of mutations in both the receptor and the α -toxin. The research focuses on the binding effects of point mutations on the α -subunit and on the γ and δ subunits defined based on the homology model. Analysis of complementary binding sites will further highlight the role of cation- π and long range Coulombic interactions in binding the toxin as well as further characterize the orientation of the disulfide loops of the α -toxin with respect to the $\alpha\gamma$ and $\alpha\delta$ interfaces on the nAChR.

Nmml mutations K47E and R36E were constructed from mutagenic oligonucleotides by subcloning cDNAs into the PEZZ18 expression vector. Introduction of a charge difference at positions 47 shows site selectivity between the $\alpha\delta$ and $\alpha\gamma$ sites whereas K47A showed identical binding at the two sites. The mutation R36E, located on loop II and ~7 Å from R33, shows two orders of magnitude shift at the $\alpha\delta$ -site and four orders of magnitude shift at the $\alpha\gamma$ -site.

Double mutant binding assays involving R36E and K47E and the α -subunit residues outlined above have been conducted. Ther-

modynamic mutant cycle analyses for the mutant pairs have assessed the effect of receptor mutations on the direct binding to the α -toxin versus conformation changes in the receptor or the ligand. Coupling energies were calculated from the difference in free energy of binding caused by the single mutation relative to its wild-type. The coupling energies for the mutant pair R36E–V188D showed simple additivity of free energy for the single mutations, which suggests that the residues are not linked or interacting. Whereas a decrease in affinity of 2.6 and > 1.5 kcal/mol at the $\alpha\delta$ and $\alpha\gamma$ sites, respectively, was observed for the R33E–V188D mutant pair⁶. The R36E–V188K double mutant did show, however, an increase in affinity of ~3.0 kcal/mol at the $\alpha\gamma$ site, which is 1.5 kcal/mol greater increase in affinity than that observed for R33E–V188K⁶. These initial results suggest that interaction between positions 36 on the toxin and position 188 on the receptor is not a simple case of coulombic attraction and repulsion. This presentation will further analyze binding energetics for the pairwise interactions involving R36E and K47E with the α -subunit mutations at positions 188, 190, 197, and 200 as well as with δ - and δ -subunit mutations such as the pairs γ Ser111/ δ Tyr113 and γ Phe172/ δ Ile178 located amino and carboxy-terminal to the conserved disulfide loop in the receptor subunits.

References

1. Tsigelny, I., Sugiyama, N., Sine, S. M. & Taylor, P. (1997) *Biophys. J.* 73, 1–15.
2. Ackermann, E. J. & Taylor, P. (1997) *Biochemistry* 36, 12836–12844.
3. Sine, S. & Taylor, P. (1979). *J. Biol. Chem.* 254, 3315–3325.
4. Sine, S. M., Kreienkamp, H.-J., Bren, N., Maeda, R. & Taylor, P. (1995) *Neuron* 15, 205–211.
5. Trembeau, O., Lemaire, C., Drevet, P., Pinkasfeld, S., Duncanson, F., Boulain, J. C. & Menez, A. (1995) *J. Biol. Chem.* 270, 9362–9369.
6. Ackermann, E. J., Ang, E. T.-H., Kanter, J., Tsigelny, I. & Taylor, P. (1998) *J. Biol. Chem.* 273, 10958–10964.

68

Effect of differentiation on electrically-evoked transmitter release from NG108-15 cells loaded with exogenous acetylcholine

M. Malo, J. Bruner, J. Stinnakre, L. Prado de Carvalho

Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, 91198 Gif-sur-Yvette, cedex, France

NG108-15 cells synthesize acetylcholine (ACh) and form functional cholinergic synapses after cell differentiation by dibutyryl cyclic AMP (dbcAMP) (Nirenberg et al., 1983; Zhong et al., 1995). DbcAMP however has no effect on ACh release from NG108-15 cells preloaded with ACh and evoked by the calcium ionophore A23187 (Israël et al., 1994).

This lack of effect could be due to the fact that the calcium ionophore bypasses the activation of voltage activated calcium channels. Indeed, calcium channel expression in NG108-15 cells is modulated by cell differentiation: non-differentiated cells have only rapidly inactivating T-type channels, whereas slowly or non-inactivating N- and L-type channels appear after cell differentiation (Eckert et al., 1990; Kasai and Neher, 1992).

The aim of this work was to compare ACh release from differentiated and non-differentiated NG108-15 cells preloaded with

ACh, in a situation where release was evoked by an electrical stimulation. Under these conditions calcium influx should occur through the existing voltage-activated calcium channels. Thus, if calcium channels were implicated one would expect less release from non-differentiated cells.

Cell differentiation was obtained by treatment with dbcAMP or with prostaglandine (PGE1) and theophylline. Under our experimental conditions both treatments greatly enhance the expression of N- and L-type channels in NG108-15, the second treatment being more efficient.

For release experiments, all cells were incubated with 40 mM ACh, in the presence of a cholinesterase inhibitor, for 24 h before the experiments. *Xenopus laevis* myocytes were used as ACh detector cells (Dan et al., 1994; Falk-Vairant et al., 1996). NG108-15 cells were removed from their culture dishes, transfer-

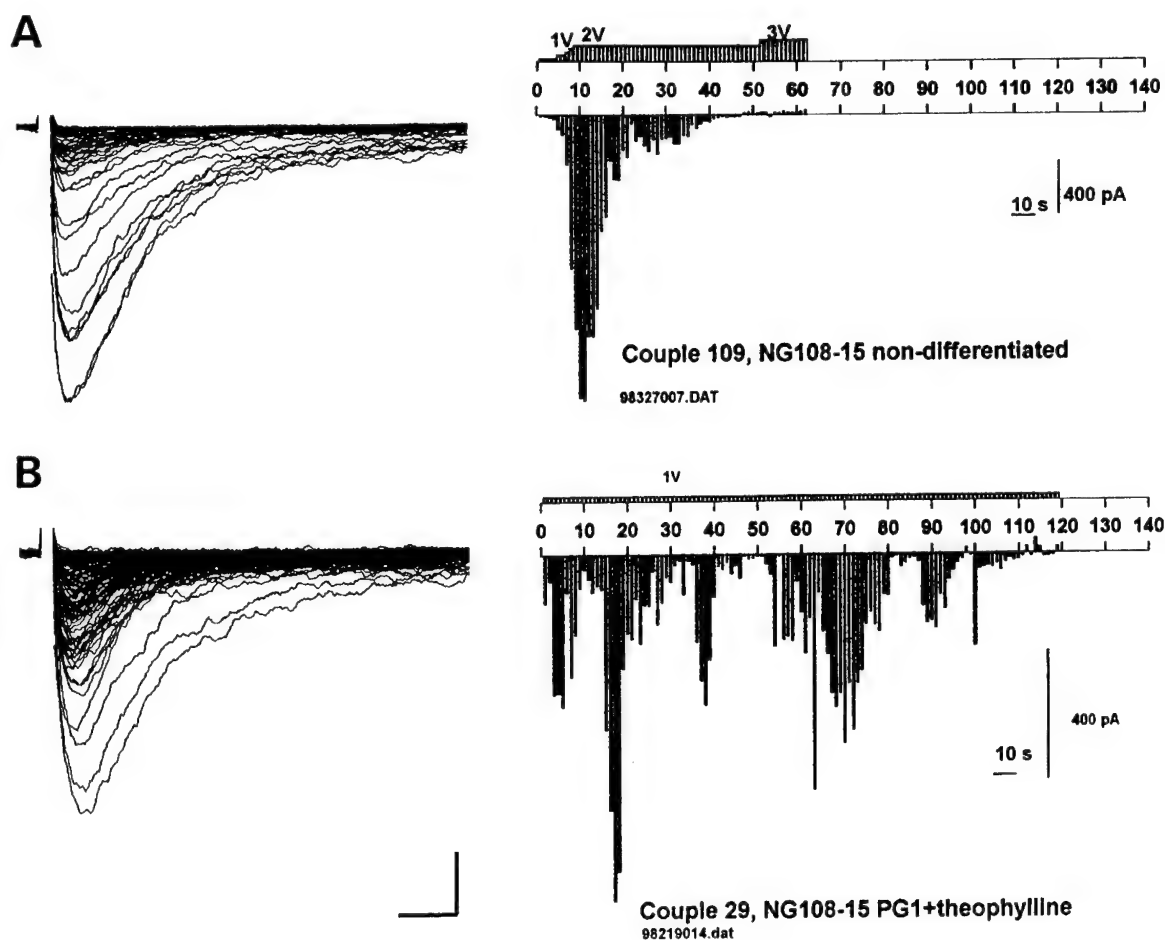


Figure 1. Electrically evoked ACh release by NG108-15 cells detected by *Xenopus* myocytes. **A.** Non-differentiated NG108-15. **B.** NG108-15 treated with prostaglandine PGE1 (10 μ M) and theophylline (1 mM) for 3 days. Left column: superimposed responses of the myocyte during a train of stimulations (1–3 V, 2 ms, 0.4 Hz) Scale: vertical 500 pA (top), 250 pA (bottom). Horizontal 10 ms. Right column: Amplitude of individual responses shown on the left column, graphed in chronological order. Voltage applied to the stimulation pipette is indicated on the top. All cells were loaded with exogenous ACh. Please note the different scales in **A** and **B**.

red into the recording dish and placed in the vicinity of a myocyte, with the help of a suction electrode that was also used to stimulate the cell. Myocytes membrane currents were recorded under voltage clamp in whole cell configuration.

ACh-loaded non-differentiated NG108-15 cells were found to release ACh upon electrical stimulation, and several evoked responses could be observed from the same cell (*figure 1*). When external calcium was replaced by 10 mM Mg^{2+} (in the presence of EGTA), release was no longer observed (not shown).

DbcAMP or PGE1 + theophylline treatments affected neither the amplitude nor the duration of electrically evoked release; these treatments, however, increase the total number of responses observed with a given cell (*figure 1*).

The present results demonstrate that non-differentiated cells loaded with ACh are able to release it under electrical stimulation as they were under calcium ionophore challenge. Thus, calcium

sources other than voltage-activated calcium channels must be involved. M. Malo was supported by an AFM fellowship.

References

- Dan Y, Hong H-j, Poo M-m (1994) Evoked neuronal secretion of false transmitters. *Neuron* 13,909–917.
- Eckert R, Hescheler J, Krauwurst D, Schultz G, Trautwein W (1990) Calcium current of neuroblastoma glioma hybrid cells after cultivation with dibutyryl cyclic AMP and nickel. *Pflügers Arch* 417, 329–335.
- Falk-Vairant JF, Corrèges P, Eder-Colli L, Salem N, Roulet E, Bloc A, Meunier F, Lesbats B, Loctin F, Synguelakis M, Israël M, Duman Y (1996) Quantal acetylcholine release induced by mediator transfection. *Proc Natl Acad Sci USA* 93, 5203–5207.
- Israël M, Lesbats B, Synguelakis M, Joliot A (1994) Acetylcholine accumulation and release by hybrid NG108-15, glioma and neuroblastoma cell- Role of a 16 kDa membrane protein in release. *Neurochem Int* 25, 103–109.

- Kasai H and Neher E (1992) Dihydropyridine-sensitive and omega-conotoxin-sensitive calcium channels in a mammalian neuroblastoma-glioma cell line *J Physiol (London)* 448, 161-188.
- Nirenberg M, Wilson S, Higashida H, Rotter A, Krueger K, Busis N, Ray R, Kenimer JG, Adler M (1983) Modulation of synapse for-

mation by cyclic adenosine monophosphate. *Science* 222, 794-799.

- Zhong ZG, Misawa H, Furuya S, Kimura Y, Noda M, Yokoyama S Higashida H (1995) Overexpression of choline acetyltransferase reconstitutes discrete acetylcholine release in some but not all synapse formation-defective neuroblastoma cell. *J Physiol (Paris)* 89, 137-145.

69

Tetrameric assembly and peripheral site-occluding loop of mouse acetylcholinesterase

P. Marchot^a, P. Taylor^b, J.R. Kanter^b, P.E. Bougis^a, Y. Bourne^c

^aCNRS UMR 6560 Ingénierie des Protéines, Institut Fédératif de Recherche Jean Roche, Université de la Méditerranée, Marseille, France;

^bDepartment of Pharmacology 0636, University of California at San Diego, La Jolla, CA, USA;

^cCNRS UPR 9039 Architecture et Fonction des Macromolécules Biologiques, Institut de Biologie et Microbiologie Structurale, Marseille, France

In mammals, the molecular forms of the cholinesterases are primary determinants of their tissue distribution and disposition within a cell; association of subunits also governs the turnover of the enzyme. The predominant form of acetylcholinesterase (AChE) in the central nervous system is an amphiphilic tetramer anchored to the membrane via a hydrophobic, non-catalytic subunit, whereas at the neuromuscular junction it is an asymmetric form containing one to three tetramers and associated with the basal lamina via a collagen-like subunit [1]. Abnormal associations of AChE arise in dementias of the Alzheimer type, where a significant and selective loss of the amphiphilic AChE tetramers is observed [2]. However, there is currently little structural information about the molecular determinants involved in association of subunits into tetramers and of tetramers with membrane-anchor subunits.

The crystal structure of a recombinant AChE from mouse (mAChE) in a complex with the peptidic inhibitor fasciculin (Fas2) provided the first template for the mammalian cholinesterases [3]. Slight differences in conformation were observed in the structure of Fas2-associated mAChE, compared with uncomplexed *Torpedo californica* AChE [4]. However, the limitations from comparing two different enzyme species may have precluded distinction of the species-related differences from the changes in conformation brought about by fasciculin binding. In addition, currently available crystallographic analyses of AChE reveal structures in which the entrance of the active site gorge is either occluded by a symmetry-related molecule or perfectly sealed by the inhibitor fasciculin [3-7], therefore precluding structural analysis of the peripheral anionic site region in its free state. A crystal structure of mAChE in the absence of bound fasciculin might therefore yield a conformational state resembling more closely that of the active enzyme in solution.

The crystal structure of mAChE was solved by molecular replacement using the mAChE coordinates extracted from the 3.2 Å resolution structure of the Fas2-mAChE complex as a search

model; it was refined to 2.9 Å resolution with a R-factor and a R-free value of 21.5% and 24.5%, respectively, in the 15 Å to 2.9 Å resolution range.

This structure provides a significantly improved accuracy in the positions of the main and side chains of the mAChE molecule than the Fas2-mAChE structure, confirms some distinctive features of the mouse enzyme compared with *Torpedo* AChE, and permits direct comparison of a free and an occluded peripheral anionic site within the same crystal unit. Most importantly, this structure highlights certain determinants at the surface of mAChE that could participate in formation of oligomers upon assembly in normal and pathological states, allosteric modulation of catalysis, or surface interactions involved in non-cholinergic AChE functioning. This work was supported by USPHS and DAMD grants to P.T., a NSF-CNRS collaborative project to P.T. and P.M., the AFM to P.M. and P.E.B., and the European Union to Y.B. and P.M.

References

- [1] Massoulié J, Pezzementi L, Bon S, Krejci E & Valette FM (1993) *Prog. Neurobiol.* 41, 31-91
- [2] Schegg KM, Harrington LS, Nielsen S, Zweig RM & Peacock JH (1992) *Neurobiology of Aging* 13, 697-704
- [3] Bourne Y, Taylor P & Marchot P (1995) *Cell* 83, 503-512
- [4] Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Tokar L & Silman I (1991) *Science* 253, 872-879
- [5] Harel M, Schalk I, Ehret-Sabatier L, Bouet F, Goeldner M, Hirth C, Axelsen PH, Silman I & Sussman JL (1993) *Proc. Natl. Acad. Sci. USA* 90, 9031-9035
- [6] Axelsen PH, Harel M, Silman I & Sussman JL (1994) *Protein Sci.* 3, 188-197
- [7] Harel M, Kleywegt GJ, Ravelli RBG, Silman I & Sussman JL (1995) *Structure* 3, 1355-1366
- [8] Raves ML, Harel M, Pang YP, Silman I, Kozikowski AP & Sussman JL (1997) *Nature Struct. Biol.* 4, 57-63

70

Structural discrimination between the two agonist binding sites of the *Torpedo* nicotinic acetylcholine receptor using time resolved fluorescence

K. Martinez^a, P.J. Corringer^b, F. Merola^a, J.-P. Changeux^b

^aLURE, Bât 209 D, Centre Universitaire Paris-Sud, B.P. 34, 91898 Orsay cedex;

^bUnité de Neurobiologie Moléculaire, Institut Pasteur, 25, rue du Dr-Roux, 75724 Paris cedex 15, France

Muscle type nicotinic acetylcholine receptors (nAChR) are heteropentamers $\alpha 2\beta\gamma\delta$. They carry two binding sites for acetylcholine and competitive effectors at the α/γ and α/δ subunit interfaces. To investigate for structural differences between the two binding sites, we used time resolved fluorescence (TRF).

TRF consists of exciting a fluorescent probe during an extremely short time, and measuring the time required by this molecule to reemit light (nanosecond time range). The re-emission speed depends on both the nature and chemical environment of the fluorescent probe. In the present work, we used the single photon counting method¹⁻³, which establishes the re-emission probability of a photon versus time with very high accuracy. Mathematical analysis was performed using the maximum entropy method^{4,5} to give the fluorescence lifetime distribution, each lifetime putatively representing a particular state of the probe.

We studied the interactions between the fluorescent probe dansyl-C6-choline⁶ (Dns-C6-Cho, excitation at 330 nm and emission at 520 nm) and the nAChR from *Torpedo marmorata*, solubilized in CHAPS. The lifetime distribution of free Dns-C6-Cho in buffer containing CHAPS is quasi monoexponential with a lifetime of 3.7 ns. In contrast, the distribution of Dns-C6-Cho in the presence of the receptor is multi-exponential, with lifetimes of 0.25, 1.5, 5 and 18 ns (mean lifetime of 9 ns). Preincubation of the receptor with α -bungarotoxin results in a distribution corresponding to free Dns-C6-Cho, showing that the signal is specific for the competitive agonist binding sites.

To detect putative differences in binding affinity and fluorescence fingerprint of the two binding sites, we measured the fluorescence decay at various occupation ratios of receptor by Dns-C6-Cho. No significant differences were found from 20 to 100% occupation of the binding sites, indicating that Dns-C6-Cho

displays similar binding affinities for the two sites or that the microenvironment of Dns-C6-Cho in both sites is similar.

In order to discriminate between these two hypotheses, we used the α -conotoxin M1^{7,8}, which has a 20-fold higher affinity for the α/γ agonist binding site as compared to the α/δ binding site on our receptor preparation. We studied the fluorescence decay of bound Dns-C6-Cho at various occupation ratios of the α -conotoxin M1 on the receptor. Preliminary experiments show different distributions at low and high occupation ratios, indicating that Dns-C6-Cho displays different fluorescent distributions depending on the binding sites. These data suggest that Dns-C6-Cho remains in different microenvironments when bound to the two sites, possibly due to the contributions of the γ and δ subunits.

References

- (1) Phillips, D. Drake, R. C.; O'Connor, D. V.; Christensen, R. L. *Analytical Instrumentation* 1985, 14, 267-292.
- (2) Wahl, P. In *New techniques in biophysics and cell biology*; H. Pain and B. Smith, Ed.; Wiley: London, 1975; Vol. 2; pp 233-285.
- (3) Yguerabide, J. *Methods in Enzymology* 1972, 26, 498-578.
- (4) Livesey, A. K.; Skilling, J. *Acta Crystallographica, Section B* 1985, A41, 113-122.
- (5) Livesey, A. K.; Brochon, J. C. *Biophysical Journal* 1987, 52, 693-706.
- (6) Waksman, G.; Changeux, J. P.; Roques, B. P. *Molecular pharmacology* 1980, 18, 20-27.
- (7) Hann, R. M.; Pagan, O. R.; Eterovic, V. A. *Biochemistry* 1994, 33, 14058-14063.
- (8) Groebe, D. R.; Dumm, J. M.; Levitan, E. S.; Abramson, S. N. *Molecular Pharmacology* 1995, 48, 105-111.

71

Is nicotine sharing common molecular and anatomical substrates with cocaine?

A.M. Mathieu-Kia, M. Rogard, M.J. Besson

Laboratoire de Neurochimie Anatomie, Institut des Neurosciences, UMR 7624, 9, quai Saint-Bernard, 75005 Paris, France

Nicotine and cocaine are both psychostimulant drugs which activate dopamine (DA) neurons in the ventral mesencephalon. These neurons project massively to the striatal complex, the dorsal part (or striatum) which is involved in movement co-ordination and the ventral part (or nucleus accumbens, NAc) which is part of the limbic system. To investigate possible common molecular targets between these two drugs we have examined the effects produced by repeated injections (3 times a day during 15 days) of either nicotine or cocaine on peptide expression in the striatal complex and by a single or repeated injections on immediate early gene expression in various cerebral regions.

In the dorsal striatum, cocaine increased mRNAs encoding for substance P (PPT-A) and dynorphin (PPDYN) whereas nicotine did not change the levels of these mRNAs. The mRNA encoding for preproenkephalin (PPE), remained unchanged with both treatments. Similarly, in the ventral striatum (or NAc), nicotine did not affect the peptide mRNA expression except in the shell where it produced a decrease of PPDYN mRNA. In contrast, cocaine treatment induced increases of PPT-A mRNA in the core and PPDYN and PPE mRNAs in the rostral pole of the NAc. Thus in the striatal complex, peptides which are expressed by the great majority of neurons did appear to be common substrates for these

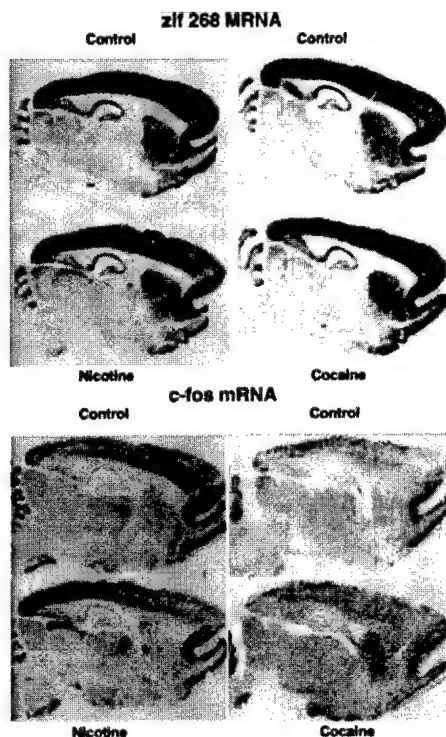


Figure 1. Rats were treated with nicotine (0.4 mg/kg, s.c.) or cocaine (12 mg/kg, i.p.) and killed 40 min later. Brains were cut sagittally and sections were processed for zif 268 and c-fos mRNA using 35S riboprobes. It can be noted that nicotine mainly induced zif 268 and c-fos mainly in the superior colliculus and associated structures as well as some thalamic nucleus (in particular the antero-ventral nucleus and the septum). In contrast, cocaine produced an induction of zif 268 and c-Fos mRNA mainly in the striatal complex.

two drugs; their expression was preferentially changed by cocaine (Mathieu-Kia et al., 1998).

The induction of immediate early genes (c-fos and zif 268) which can be considered as markers of changes in neuronal ac-

tivity was also examined. Forty minutes after an acute injection of nicotine (0.4 mg/kg, s.c.) c-fos and zif 268 mRNA were increased in visual, visuo-motor and retino-limbic structures as well as in amygdala and septum but not in the dorsal striatum. In contrast, c-fos and zif 268 mRNAs were markedly increased but only in the striatal complex following an acute cocaine injection (12 mg/kg, i.p.) (see figure 1).

The c-Fos protein analysed 90 min after an acute (0.4 mg/kg) and the last injection of a chronic nicotine (0.4 mg/kg, 3 times a day for 15 days, s.c.) administration showed a pattern of induction similar to that found with c-fos mRNA analysis and in addition pointed to the involvement of various cortical areas. The acute versus chronic treatment comparison indicated a maintenance of c-Fos inducibility and in some structures a larger recruitment of c-Fos positive neurons after chronic nicotine injections (Mathieu-Kia et al., 1998). The persistence of a c-Fos induction was generally not observed after a chronic cocaine treatment which instead generates chronic fos related antigens (Chen et al., 1995; Nye et al., 1997).

While the striatal complex appears as a preferential target for cocaine, visual, retino-limbic and limbic areas are preferential targets for nicotine indicating that nicotine and cocaine share few common anatomical substrates.

References

- Chen J, Kelz MB, Hope BT, Nakabeppu Y, Nestler EJ (1997) Chronic Fos-related antigens: stable variants of deltaFosB induced in brain by chronic treatments. *J Neurosci* 17:4933-4941
- Mathieu-Kia AM, Besson MJ (1998) Repeated administration of cocaine, nicotine and ethanol: effects on preprodynorphin, preprotachykinin A and preproenkephalin mRNA expression in the dorsal and the ventral striatum of the rat. *Brain Res Mol Brain Res* 54:141-151
- Mathieu-Kia AM, Pages C, Besson MJ (1998) Inducibility of c-Fos protein in visuo-motor system and limbic structures after acute and repeated administration of nicotine in the rat. *Synapse* (in press).
- Nye HE, Hope BT, Kelz MB, Iadarola M, Nestler EJ (1995) Pharmacological studies of the regulation of chronic FOS-related antigen induction by cocaine in the striatum and nucleus accumbens. *J Pharmacol Exp Ther* 275:1671-1680

72

Artificial toxins to explore new receptors?

A. Ménez^a, G. Mourier^a, P. Kessler^a, F. Ducancel^a, D. Bertrand^c, P.J. Corringer^b, J.P. Changeux^b, D. Servent^a

^aDépartement d'Ingénierie et d'Études des Protéines, CEA Saclay, 91191 Gif-sur-Yvette

^bLaboratoire de Neurobiologie Moléculaire, Institut Pasteur, 75724 Paris cedex 15, France

^cDépartement de Physiologie, Faculté de Médecine, 1211 Geneva 4, Switzerland

Toxins from animals, plants and microorganisms are essential tools to discover and study receptors, and to investigate complex physiological pathways. Not surprisingly, therefore, numerous studies have been undertaken to understand how toxins work. As a result, some rules describing major properties of animal toxic proteins are now emerging which may serve as guidelines for future drug design. This presentation will essentially focus on snake toxins that block acetylcholine receptors.

Curare-mimetic toxins from elapid and hydrophiid snakes are usually classified as short-chain toxins (60–62 residues and four disulphide bonds) and long-chain toxins (66 residues and 4/5 disulphide bonds). They all block acetylcholine receptors with high affinity and adopt a similar architecture, called the 'three-fingered' fold, which consists of three adjacent loops rich in β -pleated sheet protruding from a small globular core where four disulphide bonds are conserved. Using site-directed mutagenesis, we identified the functional residues by which a short-chain toxin binds to acetylcholine receptor from *Torpedo marmorata*. The site includes at least ten residues spread on the three loops. Approximately half of them, mostly located on the second and third loops, are highly conserved in both short-chain and long-chain toxins whereas the others, mostly located on the first loop are clearly variable. These observations suggest that the general propensity of curare-mimetic toxins to bind to acetylcholine receptors may be associated with both a conserved functional core which may provide toxins with a minimal affinity for most receptor subtypes and with additional variable residues which may provide individual toxins with high affinity toward various receptor subtypes.

In this respect, we found it striking that only long-chain curare-mimetic toxins, such as α -bungarotoxin or α -cobratoxin, possess five additional residues cyclized by a disulphide bond,

located at the tip of the second loop. This additional small loop is unnecessary for a curare-mimetic toxin to bind with high affinity to Torpedo acetylcholine receptor. Recently, however, we discovered a specific function associated with this intriguing loop. Thus, it was observed that long-chain toxins have the capacity to bind to the $\alpha 7$ neuronal receptor with high affinities ranging from 1 to 10 nM, whereas short-chain toxins bind to this receptor with much lower affinities ranging between 2 and 22 μ M. To demonstrate that the small extra loop of long-chain toxins is directly associated with their high affinities for $\alpha 7$ -type receptors, we synthesized three categories of derivatized curare-mimetic toxins. First, we reduced selectively the additional disulphide bond of a long-chain toxin and modified its resulting free cysteines by specific reagents. The derivatized toxin displayed a considerably reduced affinity for $\alpha 7$ receptor. Second, we replaced the two extra half-cystines of the long-chain α -cobratoxin and α -bungarotoxin by serine residues and observed that their affinities for $\alpha 7$ decreased 25- and 35-fold, respectively. Third, we introduced an extra disulphide bond into a short-chain toxin having low affinity ($K_d = 2 \mu$ M) for $\alpha 7$ and found that the resulting chimeric short-chain toxin displayed a 20-fold higher affinity for this receptor. Since all these derivatized toxins for the Torpedo receptor possess virtually unchanged affinities as compared to their parent toxins, we conclude that the extra loop is a key element, probably not the only one, that is responsible for the capacity of curare-mimetic toxins to discriminate between muscular and neuronal receptors. This finding may open new perspectives to engineer artificial toxins with appropriate affinities for new acetylcholine receptor subtypes.

73

Neuronal nicotinic acetylcholine receptors: The importance of the host cell type in heterologous expression studies

N.S. Millar, S.T. Cooper, P.C. Harkness, S.J. Lansdell, I.J. Parsons

Wellcome Laboratory for Molecular Pharmacology, Department of Pharmacology, University College London, London, WC1E 6BT, UK

Several lines of evidence have led us to conclude that the folding, subunit assembly, intracellular distribution and functional properties of neuronal nicotinic acetylcholine receptors (nAChRs) can be influenced by the choice of host cell type used for heterologous expression studies.

We have found that the folding, assembly and cell-surface expression of the homo-oligomeric neuronal nAChR $\alpha 7$ and $\alpha 8$ subunits are strongly dependent upon the host cell type (Cooper and Millar, 1997; Cooper and Millar, 1998), an observation which has been supported by recent studies by other groups (Blumenthal et al., 1997; Kassner and Berg, 1997; Chen et al., 1998). Our evidence for subunit misfolding is based, in particular, upon the absence of binding of nicotinic radioligands, such as α -bungarotoxin, and conformation-sensitive monoclonal antibodies.

We have now extended these studies by examining the distribution of the $\alpha 7$ subunit by fluorescent confocal microscopy in micro-injected cultured primary neuronal cells and polarized epithelial cells. These new studies have further strengthened our belief that the folding and efficient cell surface expression of this protein is critically dependent upon the host cell type.

Achimeric $\alpha 7^{(V201)}/5HT_3$ subunit (which contains the N-terminal domain of $\alpha 7$ and C-terminal domain of the serotonin receptor 5-HT₃ subunit; Eiselé et al., 1993) is expressed very efficiently in cell lines in which $\alpha 7$ is misfolded (Cooper and Millar, 1998). Whereas the $\alpha 7$ (and $\alpha 8$) subunit appears to be misfolded when expressed in several cell types, the $\alpha 7^{(V201)}/5HT_3$ (and $\alpha 8^{(I201)}/5HT_3$) chimera is expressed efficiently on the surface of all cell types we have examined.

We believe that the cell-specific differences in the folding and transport of $\alpha 7$ (and $\alpha 8$) are not due simply to a requirement for these subunits to co-assemble with other nAChR subunits which may be expressed endogenously in cells such as primary SCG neurons or established cell lines such as SH-SY5Y and PC-12. This conclusion is supported by the observation that functional homo-oligomeric nAChRs are formed when $\alpha 7$ is expressed in several non-neuronal cell types, including the *Xenopus* oocyte (Couturier et al., 1990), rat pituitary GH4C1 cells (Quik et al., 1997), and some (Gopalakrishnan et al., 1995; Ragozzino et al., 1997), but not in other (Cooper and Millar, 1997), isolates of human kidney cells.

While the dramatic cell-specific differences in folding described above may be restricted to homo-oligomeric type nAChRs such as $\alpha 7$ and $\alpha 8$, we have also found recently that the functional properties of hetero-oligomeric recombinant neuronal nAChRs composed of $\alpha 3\beta 4$ subunits differ in both single channel conductance and relative agonist potency when expressed in either *Xenopus* oocytes or a mammalian cell line (Lewis et al., 1997). Our data indicate that the functional properties of recombinant channels expressed in cultured mammalian cell lines resemble more closely native nAChR channels (e.g., those expressed in the rat superior cervical ganglion) than do recombinant channels expressed in *Xenopus* oocytes. Although it is not yet clear what is responsible for either these host cell-dependent differences in ion channel properties or the cell-specific folding of the $\alpha 7$ subunit (described above and in Cooper and Millar, 1997; Cooper and Millar, 1998), these findings help to illustrate the critical importance of the host cell environment.

We have also shown recently that the folding of *Drosophila* nAChRs is temperature-sensitive (Lansdell et al., 1997), as has been reported previously with the Torpedo nAChR (Paulson and Claudio, 1990). In contrast, we have shown that several *Drosophila* G-protein-coupled receptors, such as a muscarinic AChR (Millar et al., 1995), a dopamine receptor (Han et al., 1996) and an octopamine receptor (Han et al., 1998) form functional receptors when expressed in either mammalian cells (at 37 °C) or in a *Drosophila* cell line (at 25 °C). The functional expression of *Drosophila* GABA-gated ion channels in a *Drosophila* cell line (Millar et al., 1994; Buckingham et al., 1996) together with the absence of reports of the successful expression of this or other invertebrate ligand-gated channels in mammalian cell lines suggest that the temperature-sensitive folding seen with *Drosophila* nAChRs may reflect a phenomenon which is common to other ligand-gated ion channels.

References

- Blumenthal, E. M., Conroy, W. G., Romano, S. J., Kassner, P. D. and Berg, D. K. (1997). Detection of functional nicotinic receptors blocked by α -bungarotoxin on PC12 cells and dependence of their expression on post-translational events. *J. Biol. Chem.* 15, 6094–6104.
- Buckingham, S. D., Matsuda, K., Hosie, A. M., Baylis, H. A., Squire, M. D., Lansdell, S. J., Millar, N. S. and Sattelle, D. B. (1996). Wild-type and insecticide-resistant homooligomeric GABA receptors of *Drosophila melanogaster* stably expressed in a *Drosophila* cell line. *Neuropharmacol.* 35, 1393–1401.
- Chen, D., Dang, H. and Patrick, J. W. (1998). Contributions of N-linked glycosylation to the expression of a functional $\alpha 7$ -nicotinic receptor in *Xenopus* oocytes. *J. Neurochem.* 70, 349–357.
- Cooper, S. T. and Millar, N. S. (1997). Host cell-specific folding and assembly of the neuronal nicotinic acetylcholine receptor $\alpha 7$ subunit. *J. Neurochem.* 68, 2140–2151.
- Cooper, S. T. and Millar, N. S. (1998). Host cell-specific folding of the neuronal nicotinic receptor $\alpha 8$ subunit. *J. Neurochem.* 70, 2585–2593.
- Couturier, S., Bertrand, D., Matter, J. M., Hernandez, M. C., Bertrand, S., Millar, N., Valera, S., Barkas, T. and Ballivet, M. (1990). A neuronal nicotinic acetylcholine receptor subunit ($\alpha 7$) is developmentally regulated and forms a homo-oligomeric channel blocked by α -BTX. *Neuron* 5, 847–856.
- Eiselé, J.-L., Bertrand, S., Galzi, J.-L., Devillers-Thiéry, A., Changeux, J.-P. and Bertrand, D. (1993). Chimeric nicotinic-serotonergic receptor combines distinct ligand binding and channel specificities. *Nature* 366, 479–483.
- Gopalakrishnan, M., Buisson, B., Touma, E., Giordano, T., Campbell, J. E., Hu, I. C., Donnelly-Roberts, D., Arneric, S. P., Bertrand, D. and Sullivan, J. P. (1995). Stable expression and pharmacological properties of the human $\alpha 7$ nicotinic acetylcholine receptor. *Eur. J. Pharmacol.* 290, 237–246.
- Han, K.-A., Millar, N. S. and Davis, R. L. (1998). A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. *J. Neurosci.* 18, 3650–3658.
- Han, K.-A., Millar, N. S., Grotewiel, M. S. and Davis, R. L. (1996). DAMB, a novel dopamine receptor expressed specifically in *Drosophila* mushroom bodies. *Neuron* 16, 1127–1135.
- Kassner, P. D. and Berg, D. K. (1997). Differences in the fate of neuronal acetylcholine receptor protein expressed in neurons and stably transfected cells. *J. Neurobiol.* 33, 968–982.
- Lansdell, S. J., Schmitt, B., Betz, H., Sattelle, D. B. and Millar, N. S. (1997). Temperature-sensitive expression of *Drosophila* neuronal nicotinic acetylcholine receptors. *J. Neurochem.* 68, 1812–1819.
- Lewis, T. M., Harkness, P. C., Sivilotti, L. G., Colquhoun, D. and Millar, N. S. (1997). The ion channel properties of a rat recombinant neuronal nicotinic receptor are dependent on the host cell type. *J. Physiol.* 505, 299–306.
- Millar, N. S., Baylis, H. A., Reaper, C., Bunting, R., Mason, W. T. and Sattelle, D. B. (1995). Functional expression of a cloned *Drosophila* muscarinic acetylcholine receptor in a stable *Drosophila* cell line. *J. Exp. Biol.* 198, 1843–1850.
- Millar, N. S., Buckingham, S. D. and Sattelle, D. B. (1994). Stable expression of a functional homo-oligomeric *Drosophila* GABA receptor in a *Drosophila* cell line. *Proc. R. Soc. Lond. B.* 258, 307–314.
- Paulson, H. L. and Claudio, T. (1990). Temperature-sensitive expression of all-Torpedo and Torpedo-rat hybrid AChR in mammalian muscle cells. *J. Cell Biol.* 110, 1705–17.
- Quik, M., Philie, J. and Choremis, J. (1997). Modulation of $\alpha 7$ nicotinic receptor-mediated calcium influx by nicotinic agonists. *Mol. Pharmacol.* 51, 499–506.
- Ragozzino, D., Fucile, S., Giovannelli, A., Grassi, F., Mileo, A. M., Ballivet, M., Alema, S. and Eusebi, F. (1997). Functional properties of neuronal nicotinic acetylcholine receptor channels expressed in transfected human cells. *Eur. J. Neurosci.* 9, 480–488.

74

Probing the structure of the ligand binding site on the muscle nicotinic receptor with Waglerin peptides

B.E. Molles^a, E.F. Kline^a, S.M. Sine^b, J.J. McArdle^c, P. Taylor^a

^aDept. of Pharmacology, University of California, San Diego, La Jolla, CA 92093-0636, USA

^bDepartment of Physiology and Biophysics, Mayo Foundation, Rochester MN 55905, USA

^cDepartment of Pharmacology and Toxicology, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103-2714, USA

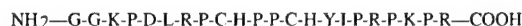
Waglerin-1 (Wtx-1) is a 22 amino acid peptide isolated from the venom of Wagler's Pit Viper, *Trimeresurus wagleri*. It acts via blockade of the nicotinic receptor at the adult mouse neuromuscular junction (nAChR); however, neonatal mice are resistant to the lethal effects of the toxin. In ¹²⁵I- α -bungarotoxin (¹²⁵I- α -Bgtx) competition experiments performed on HEK cells transfected with cDNAs for the adult form of the receptor (α , β , δ , and ϵ subunits), biphasic competition curves are produced with a K_D at the α - ϵ interface of 12.3 nM and at the α - δ interface of 21.3 μ M. In experiments performed on the embryonic form of the receptor (α , β , δ , and γ subunits), a single phase curve with a K_D of 22.0 μ M is produced, indicating that the Waglerin-1 is over 1700-fold selective for the α - ϵ interface over the α - δ or α - γ interfaces of the muscle nicotinic receptor. A series of chimeric and point-mutated γ and ϵ subunits were constructed in order to find the regions of the receptor responsible for the observed difference in affinity between the two sites and to determine which residues of the receptor were making a specific interaction with the toxin. Initial work was done on γ - ϵ chimeras in which the N-terminus from the γ -subunit was spliced to the C-terminus of the ϵ -subunit at a given junctional residue. In this series of experiments, the γ 74 ϵ chimera produced a 45-fold reduction in affinity, and the γ 103 ϵ chimera produced over 1000-fold change in affinity nearly to that found in the wild-type γ -subunit. These two experiments would indicate that there is an affinity determinant in the N-terminal 74 residues as well as between residues 74 and 103. No further decrease in affinity occurred in the γ 165 ϵ , γ 173 ϵ or γ 177 ϵ chimeras. Further smaller chimeras focusing on the region around residue 59 were constructed since this area has been found to be important for interactions with other ligands. Residues 59 and 61 of the ϵ -subunit, when mutated to the corresponding residues in γ , caused a reduction in affinity comparable to that found for the γ 74 ϵ chimera, with the individual point mutants contributing equally and additively to the affinity reduction. However, mutations at these residues in γ and δ to those found in ϵ did not reproduce a corresponding increase in affinity. The region between 74 and 103 has not yet been investigated.

In addition to the chimeras, experiments were performed on point mutants previously found to be important for determining the selectivity of the peptide toxin α -conotoxin MI. Of the three conotoxin determinants at residues 34, 111 and 173 (ϵ -subunit numbering) mutating residue Asp173 to either the Phe found in γ or the Ile found in δ caused a 500-fold and 200-fold reductions in affinity, respectively, nearly to that found in wild-type γ and δ subunits. Mutating the γ and δ subunits at the position corresponding to residue 173 to Asp caused only an 18-fold increase in affinity in δ and a four-fold increase in γ , similar to the results of the 59 and 61 position mutants. These data together indicate that residues 59, 61 and 173 are all necessary for high-affinity interaction of Waglerin-1 with the α - ϵ interface of the receptor, but not sufficient for high-affinity binding in general. Other residues that have not yet been found may be necessary for per-

mitting Waglerin-1 to interact in the high-affinity orientation at the α - γ and α - δ interfaces.

Rats are also resistant to the in vivo effects of Waglerin-1. Although this could arise from a variety of factors, we sought to determine if the differences in the primary sequences of the rat receptor subunits versus the mouse subunits were mediating this difference in in vivo effect. Initial experiments on HEK-293 cells transfected with nAChR subunit cDNAs cloned from rat indicated that the adult rat receptors have an 84-fold lower affinity at the high-affinity α - ϵ site compared to mouse, and a ~four-fold lower affinity at the α - γ and α - δ sites. This 84-fold lower affinity was linked to the rat ϵ -subunit, since HEK transfected with mouse α , β and δ subunits and the rat ϵ subunit gave the same K_D as found at the α - ϵ site of HEKs transfected with all four subunits from rat. There are 10 residues in the extracellular domain of the rat ϵ subunit that are different from mouse. Of these 10, the entire 84-fold difference in affinity can be linked to residues 59 (Asp in mouse, Glu in rat) and 115 (Tyr in mouse, Ser in rat). Changing both residues in the ϵ subunit of either species changes the affinity to that found in the wild-type subunit of the other species. When residue 59 of the mouse ϵ is changed from an Asp to an Asn, there is no change in affinity. However, changing to a Gln causes a decrease in affinity comparable to changing to a Glu, indicating that the length of the sidechain, but not the negative charge, is of primary importance for mediating the interaction of position 59 of the ϵ -subunit with Wtx-1. At residue 115, changing the Tyr in the mouse ϵ to a Phe caused no change in affinity, whereas changing the rat residue from a Ser to a Phe caused the same increase in affinity as changing to Tyr. This indicates that Waglerin-1 is having a specific interaction with the aromatic group at this position.

We have also been studying the effects on affinity that occur when specific changes in the toxin are made. The wild-type sequence of Waglerin-1 has the following sequence:



There is an intramolecular disulfide between the two cysteines. Several experiments were aimed at delineating the minimum sequence of peptide required to retain the affinity and site-selectivity of the toxin. Omitting the N-terminal four residues or the C-terminal two residues produced less than a 3.5-fold change in affinity at both sites with little change in selectivity. Omitting the C-terminal three residues caused a five-fold decrease in affinity at the low affinity α - γ and α - δ sites only. Omitting both the N-terminal and C-terminal residues ([4–18]Wtx-1) caused a 5–6-fold decrease in affinity at both sites. [7–20]Wtx-1 caused a 17-fold decrease in affinity at the high affinity site only, and [7–15]Wtx-1 gave a 713-fold decrease in affinity at the α - ϵ site, making the two sites indistinguishable. Thus, residues mediating binding at the high affinity α - ϵ site reside on both arms of the toxin, in residues 5 or 6 and residues 16, 17 or 18.

Additional changes in the toxin were made by mutating the histidines at positions 10 and 14 of the full length toxin. Changing residue 10 to a Tyr or Arg increased affinity at the α - δ site by 12.3- and 6.7-fold, respectively. Changing His-14 to a Phe caused an 8.7-fold reduction in affinity, again only at the low affinity α - δ site. All of the data taken together indicate that Waglerin-1

may interact with the receptor in a different orientation at the α - ϵ site compared to the α - δ or α - γ site. The enhanced affinity at the α - ϵ site may depend primarily on interactions from the N- and C-terminal arms of the toxin, whereas the interaction with the α - γ and α - δ sites may be conferred primarily through residues found around the disulfide loop region.

75 In vitro evidence of peroxynitrite-dependent nitration of tyrosines in presynaptic proteins: Choline acetyltransferase is a sensitive target of ONOO-, and immunodetection of nitrotyrosine coincides with inhibition of the enzyme activity

Y. Morot Gaudry-Talarmain^a, L. Lane^a, M. Israel^a, C. Ducrocq^b

^aLaboratoire de Neurobiologie Cellulaire et Moléculaire; ^bInstitut de Chimie des Substances Naturelles, CNRS, 91198 Gif-sur-Yvette cedex, France

NO has recently been proposed as a second messenger modulating cholinergic neurotransmission. in mammalian brain¹, *Aplysia* ganglion², smooth and skeletal muscle^{3,4} through prejunctional or retrograde actions. In order to study such a function at the presynaptic level, we showed previously⁵ on a model of a peri-

pheral cholinergic synapse, the nerve endings isolated from the electric organ of *Torpedo marmorata*, that NO and its derivative peroxynitrite (ONOO-) affect differently the release, the synthesis and the compartmentation of acetylcholine in synaptosomes. In the present study, we analysed further why ONOO- abolishes in

Nitration of Choline Acetyltransferase Tyrosines In Presence of Peroxynitrite

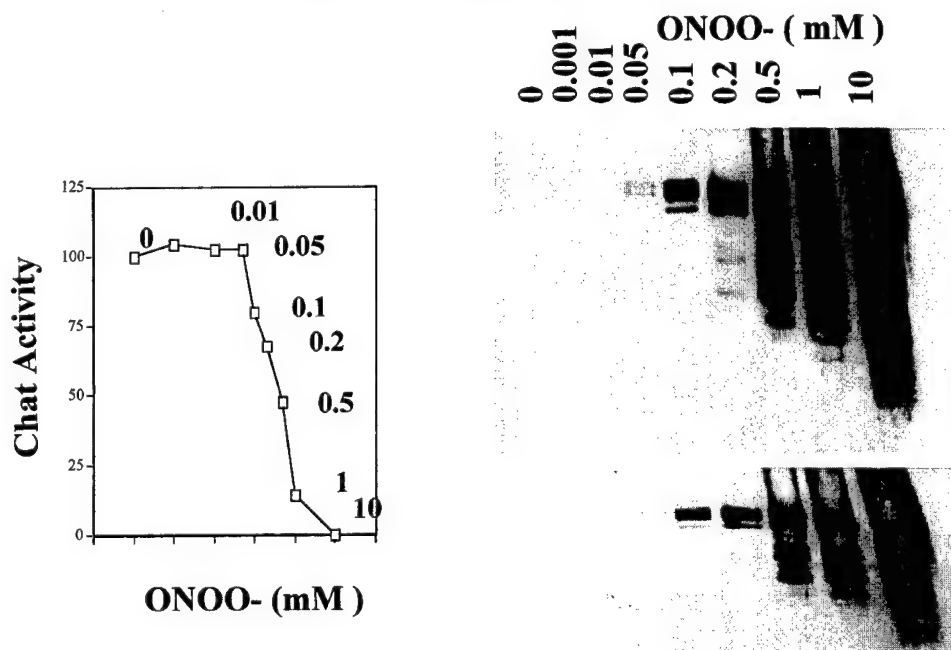


Figure 1. Left part. Dose-dependent effect of ONOO- on choline acetyltransferase activity. Choline acetyltransferase activity was measured³⁸ after 4 h preincubation time of the drug peroxynitrite (at the concentrations presented on the graph) applied directly on the enzyme. Samples were mixed with detergent (1% Triton X-100) 1 min before addition of 250 μ M eserine, 0.05% bovine serum albumin and, the substrates (1-¹⁴C)-acetyl-CoA (14 μ M), 2.5 mM choline, and 75 mM. Results of abscissa are mean of duplicate of Chat activity expressed as % of controls and are from a representative experiment in ordinate a logarithmic scale of ONOO- concentrations was used. Right part. Immunoblotting detection of nitrotyrosine on bovine brain choline acetyltransferase. Using monoclonal anti-nitrotyrosine (UBI 1/1000), the immunoreactivities of nitrotyrosine were detected by ECL-peroxidase amplifier system after Western blotting. The two parts of the figures are photographs of the same blot after two times of exposure.

a concentration-dependent manner, the synthesis of acetylcholine and the choline acetyltransferase activity of synaptosomes. With the discovery of peroxynitrite-mediated oxidation of compounds and proteins^{6,7}, our present effort was to highlight the effect of ONOO⁻ (as a function of increasing drug concentrations) on the tyrosine of neuronal proteins and in particular of choline acetyltransferase (Chat). We looked for a covalent modification of tyrosine which converts it into nitrotyrosine^{8,9}. We tried to link the changes in the protein with the enzymatic activity¹⁰.

Results

Effect of ONOO⁻ on bovine brain choline acetyltransferase activity (Chat)

Similarly to the previous experiments performed with *Torpedo* nerve endings⁵, showing a blockade of the activity of choline acetyltransferase on aliquots of synaptosomal samples treated by peroxynitrite, we investigated the effect of ONOO⁻ on a purified, and commercial source of bovine brain choline acetyltransferase. We show (figure 1, left part) that Chat activity is reduced under ONOO⁻. Inhibition starts in presence of 50 μ M ONOO⁻. Half inhibition was obtained after 500 μ M ONOO⁻, and complete inhibition was between 1 to 10 mM ONOO⁻.

Nitrotyrosine immunoreactivity detection in presynaptic proteins

Using a monoclonal anti-nitrotyrosine antibody, we then searched for immunoreactivity of proteins present in *Torpedo* synaptomes (incubated with 1 mM ONOO⁻, 4 h) after separation by SDS gel electrophoresis and transfer on nitrocellulose. With this technique of detection for nitration of tyrosines in the proteins, we observed an immunoreactivity obtained with monoclonal anti-nitrotyrosine antibody (1/1000⁵) and we noted that only a few presynaptic proteins seem to be sensitive to peroxynitrite (not shown). Among these, an immunolabeling being detected at 67–70 kDa, the protein choline acetyltransferase could be one of them.

To support this, we decided to do an in vitro treatment (with increasing concentrations of ONOO⁻) of purified bovine choline acetyltransferase from commercial source. Immunodetection of nitrotyrosine is clearly obtained after exposure to ONOO⁻. Results obtained are presented in the right parts of figure 1, and correspond to photographs of a typical ECL-treated Western blot obtained after two different time expositions. Nitrotyrosine is obtained after treatment with the concentration of 50 μ M ONOO⁻. The number of labelled bands increased progressively with higher concentrations of ONOO⁻, and a proteolysis developed further. We noted that some bands disappear at 10 mM ONOO⁻. Incubation of the blot with a polyclonal antibody against choline acetyltransferase led to a Chat immunoreactivity in presence of low concentrations of ONOO⁻ (up to 500 μ M). A loss of Chat immunoreactivity was obtained at the highest ONOO⁻ concentrations.

We also investigated the peroxynitrite-dependent nitration of choline acetyltransferase purified from non-nervous origin (human placenta) and for other proteins: acetylcholinesterase from various

sources, bovine erythrocyte Cu/Zn superoxide dismutase and cyclophilin A, an ubiquitous peptidyl prolyl cis/trans isomerase, abundant in neurons. In general, we find that ONOO⁻ (tested from 50 μ M to 10 mM) induces less immunolabelling of nitrotyrosine than for bovine brain choline acetyltransferase. Different rates of nitration of tyrosines are obtained depending on the enzyme, the source and the peroxynitrite concentrations. For placental choline acetyltransferase, electric eel acetylcholine esterase and bovine thymus cyclophilin A, immunoreactivity of nitrotyrosine was evident only at 10 mM ONOO⁻.

The present results support the notion that peroxynitrite is, in vitro, a powerful modulator of cholinergic metabolism, leading to the total loss of choline acetyltransferase activity and to a proteolysis of the protein. Further studies are in progress to detail at the level of the protein Chat itself if other residues such as cysteine, and methionine are also involved in the effect of ONOO⁻. If such a biotransformation occurs in vivo when NO and O₂⁻ are generated in excess and simultaneously, the covalent change of the choline acetyltransferase protein should be important in pathologies due to a degeneration of the cholinergic system, such as Alzheimer disease or amyotrophic lateral sclerosis in which a role for NO (and/or ONOO⁻) was previously proposed. We would like to thank to Dr. Pierre Potier, for his constant encouragement, Dr. Nicolas Morel for fruitful discussions on the immunological studies, Dr. François Marie Meunier for research on the sequence data bank of choline acetyltransferase, Dr. Seana O'Regan and Lucette Faille for help with the manuscript and the figure. Financial support was from CNRS, DRET, and Servier Laboratory.

References

1. Prast, H. and Philippu, A. (1992), *Eur. J. Pharmacol.*, 216, 139–140.
2. Mothet, J.-P., Fossier, P., Tauc, L. and Baux, G. (1996) *Proc. Natl. Acad. Sci., USA*, 93, 8721–8726.
3. Belvisi, M. G., Stretton, D. and Barnes, P.J. (1991), *Eur. J. Pharmacol.*, 198, 219–221.
4. Nakane, M., Schmidt, H.H.H.W., Pollock, J.S., Forstermann, U. and Murad, F. (1993), *FEBS Lett.*, 316, 175–180.
5. Morot Gaudry-Talarmain, Y., Moullan, N., Meunier F.A., Blanchard B., Angaut-Petit D., Faille L. and Ducrocq C (1997), *Nitric Oxide*, 1, 330–345.
6. Ohshima, H., Friesen, M., Brouet, I. and Bartsch, H. (1990), *Fundam. Chem. Toxicol.*, 28, 647–652.
7. Pryor, W.A. and Squadrito, G.L. (1995), *Am. J. Physiol.*, 268, L699–L722.
8. Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. (1991a), *J. Biol. Chem.*, 266, 4244–4250.
9. Ischiropoulos, H. and Al-Mehdi, A.B. (1995), *FEBS Lett.*, 364, 279–282.
10. Fonnum, F. (1975), *J. Neurochem.*, 24, 407–409.
11. Ducrocq C., Dendane M., Laprévotte O., Serani L., Das B.C., Bouchemal-Chibani N., Doan B.-T., Gillet B., Karim A., Carayon A. and Payen D. (1998), *Eur. J. Biochem.*, 253, 146–153.
12. Beckman, J.S., Carson, M., Smith, C.D. and Koppenol, W.H. (1993), *Nature (Lond.)*, 364, 584.

76

Photoaffinity labeling with [³H]DDF support the existence of two quaternary ammonium binding domains in human butyrylcholinesterase

F. Nachon, L. Ehret-Sabatier, M. Goeldner

Laboratoire de chimie bioorganique, UMR 7514 CNRS, Faculté de Pharmacie, Université Louis Pasteur de Strasbourg, BP 24, 67401 Illkirch cedex, France

Two types of cholinesterases (ChE) can be distinguished in vertebrates: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). These very homologous enzymes rapidly hydrolyze the neurotransmitter acetylcholine but show distinct substrate and inhibitor selectivity. X-ray structures and affinity labeling of AChE as site-directed mutagenesis of both enzymes have shown that aromatic amino acids lining the active-site gorge of ChEs are behind this different specificity. Particularly, human BuChE lacks two important aromatic residues, Phe330 and Trp279 respectively belonging to the active site and the peripheral site of Torpedo AChE. As these two residues were photolabeled by *p*-(*N,N*-dimethylamino)benzenediazonium (DDF) (1), we used this probe to study the quaternary ammonium binding domains of BuChE.

Purified human BuChE was covalently photolabeled by [³H]DDF. The photoincorporation of the probe could be fully prevented by cholinergic inhibitors such as tacrine. Following deglycosylation, alkylated BuChE was trypsinolyzed and digests were analyzed by LC/ES-MS. A comparison of tryptic fragments from labeled and unlabeled BuChE allowed to identify the tryptic peptide Y61-K103 as carrying the probe. A two-step purification procedure using respectively cation exchange chromatography and reversed phase HPLC led to highly purified labeled peptides. Microsequencing allowed to attribute radioactive signal to positions corresponding to Trp82 and Tyr332.

We propose that these labeling results reflect the occupancy of two distinct quaternary ammonium binding sites of BuChE. The photolabeling of Trp82 demonstrates that this residue shares the same feature as the homologous residue Trp84 in *Torpedo* AChE by contributing to the binding of cationic ligand through cation- π interactions. The labeling of Tyr332 supports the existence of a second quaternary ammonium binding domain in BuChE. According to the BuChE 3D-model (2) and mutagenesis results (3), we suggest that Tyr332 and Asp70 define a peripheral

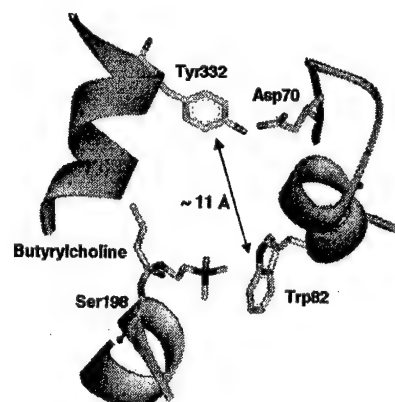


Figure 1. 3D-model of the active-site gorge of BuChE according to Harel and Sussman (2).

site of BuChE. However, the location and probably the function of this peripheral site are different from these of the AChE peripheral site.

References

1. Harel, M., Schalk, I., Ehret-Sabatier, L., Bouet, F., Goeldner, M., Hirth, C., Axelsen, P. H., Silman, I., and Sussman, J. L. (1993) *Proc. Natl. Acad. Sci. USA* 90, 9031-9035
2. Harel, M., Sussman, J. L., Krejci, E., Bon, S., Chanal, P., Mas-soulie, J., and Silman, I. (1992) *Proc. Natl. Acad. Sci. U S A* 89, 10827-10831
3. Masson, P., Froment, M. T., Bartels, C. F., and Lockridge, O. (1996) *Eur. J. Biochem.* 235, 36-48

77

Characterization of the 5'-flanking region of the mouse choline acetyltransferase gene in transgenic mice

J.M. Naciff^a, H. Misawa^b, M.M. Behbehani^a, J.R. Dedman^a

^aDepartment of Molecular and Cellular Physiology, University of Cincinnati, College of Medicine, Cincinnati, OH 45267-0576, USA

^bDepartment of Molecular Neurobiology, Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu-city, Tokyo 183, Japan

In order to identify a cholinergic tissue-specific promoter, we have analyzed the expression pattern of chimeric gene constructs consisting of 5'-sequences derived from the mouse ChAT gene and the bacterial β -galactosidase (LacZ) gene, in transgenic mice (Naciff et al., 1997, 1998). The analysis of transgenic mice bearing the β -galactosidase gene under the control of a 6417 bp segment from the 5'-untranslated region of the mouse ChAT gene (from -6371 to +46; +1 being the translation initiation site for ChAT), showed that this DNA fragment directed correct tissue-specific

expression of the reporter gene to cholinergic cells. Within this 6.4 kb DNA fragment lies an uninterrupted open reading frame, in the same transcriptional orientation as ChAT, that encodes the mouse vesicular acetylcholine (ACh) transporter (VACHT). This transporter is responsible for the translocation of cytoplasmic ACh into synaptic vesicles (Song et al., 1997). Northern blot and reverse transcription-polymerase chain reaction analysis of total RNA showed that the mouse VACHT gene is transcribed into a single mRNA isoform of ~3.0 kb, and it is expressed in spinal

cord, brain and brain stem, tissues rich in cholinergic neurons, but not in peripheral tissues such as heart, liver and kidney. The 6.4 kb DNA fragment targeted LacZ expression only to cholinergic neurons located within the cholinergic system of the CNS of transgenic mice, and also promoted the overexpression of the VAcHT gene (Naciff et al., 1997, 1998). The overexpression of VAcHT in transgenic mice did not result in any detectable modification of motor behavior, general behavior, reproductive capacity, or life span, which suggests that the cholinergic system of those animals with elevated VAcHT levels function as normally as in the wild type littermates.

We have expressed the VAcHT gene contained within the 6.4 kb fragment of the 5'-flanking region of the mouse ChAT gene in transiently transfected HeLa and Cos-7 cells. These cells normally do not express VAcHT or any other cholinergic marker. The immunolocalization of VAcHT in transfected HeLa cells (figure 1A), determined using a polyclonal antibody raised against the rat VAcHT (Chemicon International Inc., Temecula, CA, USA), shows that the expressed VAcHT is associated with vesicles located in the perinuclear region. These results demonstrate that the expressed transporter is inserted correctly in intracellular organelles delimited by membranes, where if coupled to a proton gradient could be functional. When the 6.4 kb segment from the 5'-untranslated VAcHT gene was used as spacer between a CMV promoter and a nuclear targeted synthetic concatamer gene that functionally neutralizes calmodulin (CaM; Wang et al., 1995) in the nuclei of cultured transfected cells (figure 1B), it did not interfere with the expression of this CaM inhibitor peptide.

Sequence analysis of the 6.4 kb DNA fragment from -6371 to +46, of the mouse ChAT gene (Misawa et al., 1992; Naciff et al., 1997, 1998), indicates that within the open reading frame of the VAcHT gene, there are multiple potential regulatory sequences that, acting individually or cooperatively, are required to direct specific gene expression to cholinergic neurons. However, since the overexpression of the rat VAcHT in developing *Xenopus* spinal neurons resulted in a considerable enhancement of ACh packaging at the developing synapses (Song et al., 1997), there is the possibility of modifications of the efficacy of synaptic transmission occurring in transgenic animals over-expressing the VAcHT, and this issue has to be addressed.

The use of the 6.4 kb DNA segment from the 5'-flanking region of the mouse ChAT gene, to specifically target the expression of different gene products to the cholinergic system, may require the inactivation of the VAcHT gene product, in order to eliminate possible undesired effects due to the over-expression of the VAcHT. Eliminating the expression of extra VAcHT can be accomplished by engineering a stop codon at the beginning of the coding sequence for VAcHT in the 6.4 kb fragment.

The identification of this cholinergic-specific promoter will be useful to target the expression of different gene products to

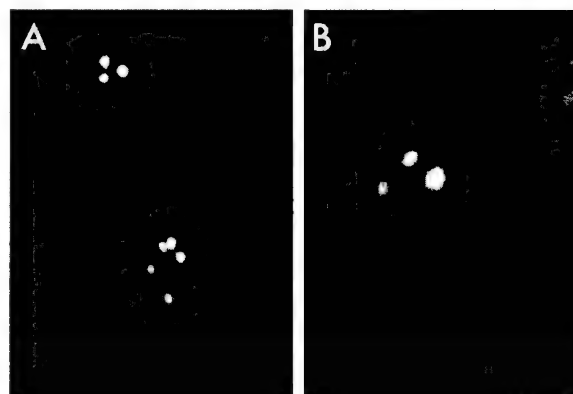


Figure 1. Immunolocalization of VAcHT and CaM-inhibitor peptide in transfected HeLa cells. HeLa cells, which do not express VAcHT or any other cholinergic marker, were transiently transfected with a chimeric gene construct consisting of a CMV promoter, the 6.4 kb DNA segment from the 5'-flanking region of the mouse ChAT gene containing the mouse VAcHT gene, and a nuclear targeted synthetic concatamer gene that functionally neutralizes calmodulin (CaM). After 24 h of transfection, cells were fixed with para-formaldehyde, permeabilized with acetone, and incubated with goat polyclonal anti-rat VAcHT (A) or rabbit anti-CaM inhibitor peptide (B) antibody, followed by fluorescein-conjugated secondary antibody.

cholinergic neurons, to understand the mechanisms of diseases characterized by malfunction of cholinergic neurons, such as Alzheimer's disease or amyotrophic lateral sclerosis, and will be valuable in the design of strategies directed to treat those neurological disorders.

References

- Misawa H, Ishi, K, Deguchi, T (1992) Gene expression of mouse choline acetyltransferase. Alternative splicing and identification of a highly active promoter region. *J Biol Chem* 267, 20392-20399
- Naciff JM, Misawa H, Dedman J.R. (1997) Molecular characterization of the mouse vesicular acetylcholine transporter gene. *NeuroReport* 8, 3467-3473
- Naciff JM, Behbehani MM, Misawa H, Dedman JR (1998) Identification and transgenic analysis of a promoter specific for cholinergic neuron expression. Submitted
- Song H-J, Ming GL, Fon E, Bellochio E, Edwards RH, Poo MM (1997) Expression of a putative vesicular acetylcholine transporter facilitates quantal transmitter packaging. *Neuron* 18, 815-826
- Wang J, Campos B, Jamieson GA Jr, Kaetzel MA, Dedman JR (1995) Functional elimination of calmodulin within the nucleus by targeted expression of an inhibitor peptide. *J Biol Chem* 270, 30245-30248

78

Involvement of protein kinases and protein phosphatases in the regulation of acetylcholine receptor gene expression

H.-O. Nghi  m, J.-P. Changeux

CNRS UA D1284, Lab. Neurobiologie Mol  culaire, D  t. Biotechnologies, Institut Pasteur, 75724 Paris cedex 15, France

Synapse formation at the neuromuscular junction (NMJ) proceeds through the enhancement of the synthesis of the acetylcholine receptor (AChR) at the NMJ and the repression of the synthesis of the extra-synaptic AChRs (outside the NMJ) by electrical activity (rev. in Changeux et al., 1990).

Protein kinases (Klarsfeld et al., 1989; Huang et al., 1992; Chahine et al., 1993) have been proposed to be involved in the repression process.

Myogenic transcription factors, which are essential for differentiation of the muscle lineage (rev. in Weintraub et al., 1991), bind to E boxes present in the AChR alpha-subunit promoter and activate transcription of the AChR gene (Piette et al., 1990).

The implication of serine/threonine protein kinases in the repression process, most probably via phosphorylation of the muscle transcription factor myogenin, has been demonstrated in chick cultures of myotubes. Myogenin (Wright et al., 1989) is phosphorylated in electrically active myotube fibers while at least partially dephosphorylated in electrically inactive (TTX-treated) myotubes (Mendelzon et al., 1994).

We further demonstrate the implication of the protein phosphatase metabolic pathway in the process of repression of the AChR gene expression. Inhibitors of protein phosphatases induce a decrease in the number of AChRs at the surface of chick myo-

tubes and prevent the upregulation of surface AChRs induced by TTX. This repression is reversible and is not associated with a dedifferentiation of the myotubes.

References

- Chahine, K.G., E. Baracchini, and D. Goldman. 1993. *J. Biol. Chem.* 268:2893-2898.
- Changeux, J.P., C. Babinet, J.L. Bessereau, A. Bessis, A. Cartaud, J. Cartaud, P. Daubas, A. Devillers-Thierry, A. Duclert, J.A. Hill, B. Jasmin, A. Klarsfeld, R. Laufer, H.O. Nghi  m, J. Piette, M. Roa, and A.M. Salmon. 1990. *Cold Spr. Har. Symp. Quant. Biol.* vol. LV. 381-396.
- Huang, C.F., J. Tong, and J. Schmidt. 1992. *Neuron* 9:671-678.
- Klarsfeld, A., R. Laufer, B. Fontaine, A. Devillers-Thierry, C. Dubreuil, and J.P. Changeux. 1989. *Neuron* 2:1229-1236.
- Mendelzon D., Changeux J.P. and H.O. Nghi  m. 1994. *Biochemistry* 33: 2568-2575.
- Piette, J., J.L. Bessereau, M. Huchet, and J.P. Changeux. 1990. *Nature* 345:353-355.
- Weintraub, H., R. Davis, S. Tapscott, M. Thayer, M. Krause, R. Ben-zra, K. Blackwell, D. Turner, R. Rupp, S. Hollenberg, Y. Zhuang, and A. Lassar. 1991. *Science* 251:761-766.
- Wright, W. E., D. A. Sassoon, and Lin., V. K. 1989. *Cell* 56:607-617.

79

Complementation of a yeast choline transport mutant by *Torpedo* electric lobe cDNAs

S. O'Regan, N. Cha, V. Matz, F.-M. Meunier

D  pt. Neurochimie, NBCM, CNRS, 91198 Gif-sur-Yvette, France

In 1992, the potassium transporter of *Arabidopsis thaliana* was cloned using an elegant paradigm based on heterologous complementation in a yeast mutant¹. Since we also knew that the choline transporter of *Saccharomyces cerevisiae* had been cloned by homologous complementation², we decided to see whether it was possible to clone the neuronal choline transporter using a similar approach. To do so, it was first necessary to establish several prerequisite conditions.

The first task was to obtain a yeast strain compatible with a yeast expression vector and a system of selection for the transformants capable of choline uptake. This was done by crossing the choline transport mutant used for homologous complementation, D308-14D (α *ctr* *ise* *leu2* *his4*, donated by Pr. S. Yamashita) with an *  ura4* strain donated along with the yeast expression vector pFL61 by Dr. F. Lacroute. The offspring were then screened for a *ctr* *ise* *  ura3* phenotype that would allow the selection of transformants in the absence of uracil at a low concentration of myo-inositol and the further selection of transformants capable of growth dependent on the uptake of extracellular choline at a high concentration of myo-inositol². The dependence of growth on choline uptake as a function of the myo-inositol concentration is regulated by the *ise* mutation; yeasts that revert to the *ise*⁺

phenotype grow well in high myo-inositol whether or not choline is present in the medium. The locus of this mutation has not yet been characterized.

The resulting strain (S208) is not very robust and most methods of transformation gave few if any transformants. Satisfactory transformation frequencies were finally achieved by using the LiAc/SS-DNA/PEG procedure³.

The last prerequisite was a yeast expression library likely to contain clones coding for the neuronal choline transporter. The *Torpedo* electric lobe was chosen as a source of mRNAs since this tissue is highly specialized for presynaptic cholinergic function and has already proven to be a good source of cholinergic clones⁴. The cDNAs were prepared and inserted into pFL61.

The experiment could now begin. In order to screen the library which originally represented 400 000 different cDNAs, up to 500 000 transformants were tested for growth under selective conditions. After elimination of *ise*⁺ revertants, about 100 clones were retained. A few of these transformed yeast were able to accumulate choline at a higher rate than the others. For these candidates, the plasmids were isolated and partially sequenced. While some of these clones could not be identified, others, such as enkephalin convertase, AKAP, p145, or cytochrome oxidase, were clearly un-

related to choline metabolism. These yeast were undoubtedly *ctr*⁺ revertants, and as such needed to be eliminated.

The second phase of screening consisted of transforming individual aliquots of yeast with the plasmids isolated from the original positive colonies. Each batch of transformants was examined for choline-dependent growth under selective conditions. None of the clones were capable of strong complementation or suppression of the *ctr* mutation. The strongest phenotype observed (about 50% of the transformants grew in selective medium) was associated with inorganic pyrophosphatase (clone 1.13) which intervenes in the incorporation of choline into phospholipids, and may be influencing choline-dependent growth in this way.

Taking into account the possibility that functional expression of a vertebrate transmembrane protein may not be optimal in the yeast, clones which had at least a weak phenotype (10–25% growth under selective conditions) were tested for choline uptake. This time large pools of transformants could be used since each transformation was done with an isolated plasmid. The results of such an experiment are shown in figure 1. Of the 24 clones tested, only clone 4.17 conferred a notable increase in choline uptake on the transformed yeast. Furthermore, choline uptake by 4.17-transformed yeast was inhibited by 10 μ M hemicholinium-3. Addition of 100 mM NaCl under hyperosmotic conditions tended to reduce choline uptake for all the clones tested and this was also seen with 4.17, although the HC-3 sensitive component was still apparent.

The sequence of clone 4.17 indicates a truncated ORF. The resulting C-terminal portion of the encoded protein has four hydrophobic regions likely to form transmembrane domains. Significant homology was found with the sequence of an unidentified *C. elegans* protein with 9–10 TMDs. This protein in turn shows a loose homology to various transporters.

The isolation of the full length clone will be necessary to further characterize the sequence and function of the expressed vertebrate protein. Studies on the distribution of the mRNA and

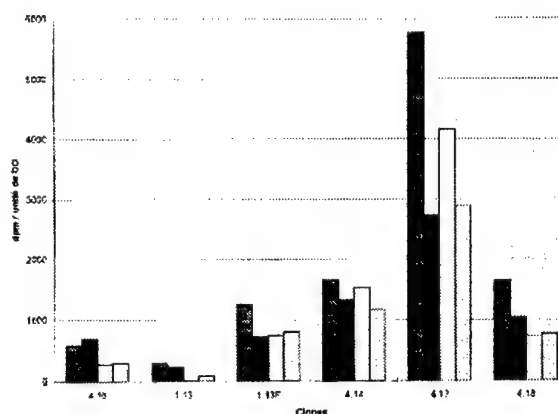


Figure 1.

of the native protein will also be needed to know if we are really on the tracks of the elusive neuronal choline transporter. We are greatly indebted to Maurice Israël for patiently allowing this story to unfold. We thank the Association Française contre les Myopathies for their financial support.

References

1. H. Sentenac, N. Bonneaud, M. Minet, F. Lacroute, J.-M. Salmon, F. Gaymard, and C. Grignon, *Nature* 256, 663–665 (1992).
2. J.-I. Nikawa, K. Hosaka, Y. Tsukagoshi, and S. Yamashita, *J. Biol. Chem.* 265, 15996–16003 (1990).
3. R.D. Gietz, R.H. Schiestl, A.R. Willems, and R.A. Woods, *Yeast* 11, 355–360 (1995).
4. H. Varoqui, M.-F. Diebler, F.-M. Meunier, J.B. Rand, T.B. Usdin, T.I. Bonner, L.E. Eiden, and J.D. Erickson, *FEBS Letters* 342, 97–102 (1994).

Binding orientation of the α -neurotoxins with the nicotinic acetylcholine receptor

H. Osaka^a, E.J. Ackerman^a, J. Kanter^a, I. Tsigelny^a, S.M. Sine^b, P. Taylor^a

^aDepartment of Pharmacology 0636, University of California, San Diego, La Jolla, California 92093, USA

^bReceptor Biology Laboratory, Department of Physiology and Biophysics, Mayo Foundation, Rochester, Minnesota 55905, USA

α -Neurotoxins, members of three fingered toxin family, may be classified into short neurotoxins (60–62 residues and four disulfide bonds; i.e., erabutoxin, Nmm neurotoxin I) and long neurotoxins (66–74 residues and five disulfide bonds; i.e., cobratoxin, α -bungarotoxin). Both specifically bind to the muscle type nicotinic acetylcholine receptor (nAChR) and for the past decades these have been the critical tool used to purify initially and then characterize the nicotinic receptor. Several other toxins, notably fasciculin and the muscarinic receptor toxins which interact with cholinergic proteins, fall into this general family. In contrast to a acetylcholine esterase-fasciculin complex whose crystal structure has been solved [1], little is known about the orientation and precise receptor residues involved in the α -neurotoxin/nicotinic receptor interaction. Studies for a short neuro-

toxin, erabutoxin b, revealed that the tips of three loops are responsible for the nAChR binding [2]. Our study for Nmm neurotoxin I (NmmI) also found three residues at the tips of three loops, K27, R33, K47, at the proximity to the binding sites [3]. Mutagenesis, labeling studies, and homology modeling to structurally known proteins [4] predict that the N-terminal extracellular 200 amino acids, especially three regions from α -subunit and four regions from non three-subunits, form the surface at the subunit interface to which various ligands bind. By focusing on amino acid sequence differences between the neuronal and muscle type, and anionic residues at α -subunit of nAChR [5], we uncovered contributions of V188, Y190, P197, D200 to the NmmI binding and the coupling between R33/K47 of NmmI and α 188–200 of nAChR by double mutant cycle analysis [6]. Because the binding

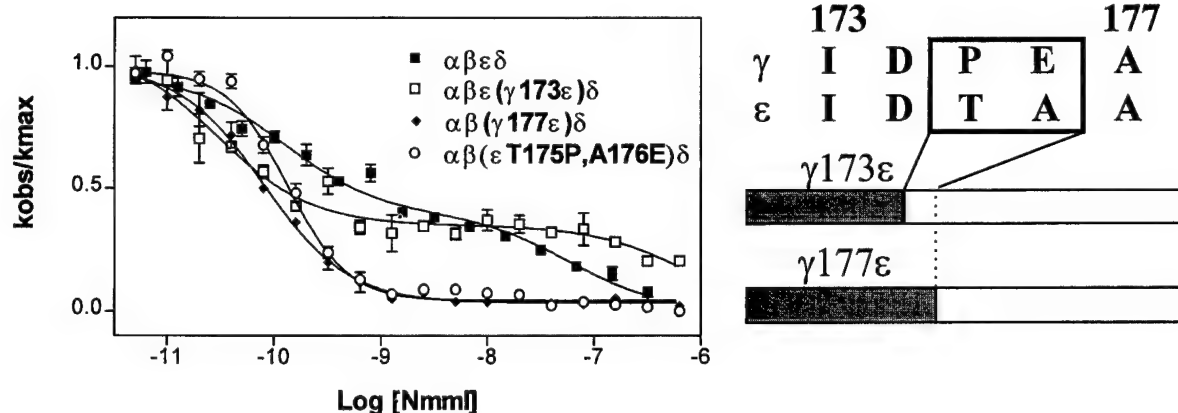


Figure 1.

surfaces are composed of the $\alpha\delta$ and $\alpha\gamma$ or $\alpha\epsilon$ subunits interfaces, we extended our work to delineate the non- α subunits/toxin interactions.

Among the determinants on the γ and δ subunits, only the L119R [7] mutation at γ subunit gave over ten-fold decrease of affinity to NmmI. Secondly, we utilized the unique $\alpha\epsilon$ interface insensitivity (K_d for $\alpha\epsilon$ ~ 0.2 mM; K_d for $\alpha\gamma$ and $\alpha\delta$ ~ 0.1 nM) to NmmI. Using subunit chimeras where the N-terminal portion of ϵ subunit is replaced with that of γ subunit, the origin of the $\alpha\epsilon$ insensitivity converged to two residues, ϵ Thr175 (Pro at γ and δ) and ϵ Ala176 (Glu at γ and δ) (figure 1). Double mutants cycle analysis combining the receptor mutants and recombinantly generated NmmI mutants disclosed a tight linkage between γ L119 at the receptor and R33 at the toxin with coupling energy of ~ 5 kcal/mol. From this linkage at γ L119 and the unique ϵ subunit resistance, together with linkages established for the α -subunit [6, 8] and a model of folding in the receptor subunits [4], the

spatial orientation of bound NmmI on the nAChR appears amenable to detailed study.

References

- [1] Bourne, Y., Taylor, P., and Marchot, P. (1995) *Cell* 83, 503–512
- [2] Trémeau, O., Lemaire, C., Drevet, P., Pinkasfeld, S., Ducancel, F., Boulain, J.-C., and Ménez, A. (1995) *J. Biol. Chem.* 270, 9362–9369
- [3] Ackerman, E. J., and Taylor, P. (1997) *Biochemistry* 36, 12836–12844
- [4] Tsigelny, I., Sugiyama, N., Sine, S. M., and Taylor, P. (1997) *Biophys. J.* 73, 52–66
- [5] Osaka, H., Sugiyama, N., and Taylor, P. (1998) *J. Biol. Chem.* 273, 12758–12765
- [6] Ackerman, E. J., Ang, E. T.-H., Kantor, J. R., and Taylor, P. (1997) *J. Biol. Chem.* 273, 10958–10964
- [7] Sine, S. M. (1997) *J. Biol. Chem.* 272, 23521–23527
- [8] Malany, S. and Taylor, P. (1998) Abstract, this volume

81

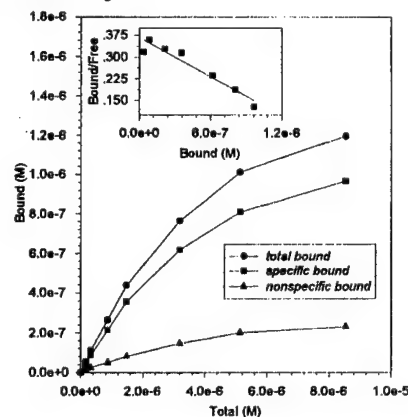
Serotonin low affinity site in the nicotinic acetylcholine receptor

M. Otero de Bengtsson, M. Biscoglio de Jiménez Bonino

Department of Biological Chemistry, Biological Chemistry and Physicochemistry Institute, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, (1113) Buenos Aires, Argentina

Serotonin (5-hydroxytryptamine, 5HT) activates one type of ligand-gated receptor channel (5HT₃) and at least 14 G-protein-coupled receptors in the central nervous system and the periphery. In addition, electrophysiological studies suggest that 5HT agents block the nicotinic acetylcholine receptor (nAChR). We obtained the first biochemical evidence of ³H-5HT binding to electric organ membranes and to the purified nicotinic re-

Figure 1. Saturation curves of the binding of ³H-5HT to the purified nAChR. The nAChR (4.5–10⁻⁴ mg/mL) was incubated with different concentrations of 3H-5HT in the presence or absence of 5HT, in the dark, at 4 °C, for 1 h. Three μ L of the samples were then dotted on a nitrocellulose membrane. The membrane was then incubated 30 min with 10 mM PBS, Triton X-100 1%, milk 5%, and washed 6 times with 100 mL of 10 mM PBS, Triton X-100 1%. The membrane was then cut in 1 cm squares which were laid on a vial with 3 mL of Xilene with 5% PPO and radioactivity was measured on a liquid scintillation counter. The figure includes data from a typical experiment. The insert shows the corresponding Scatchard analysis.



ceptor from *Discopyge tschudii* (Otero de Bengtsson and Biscoglio de Jiménez Bonino, 1997). Serotonin was used as a photo-affinity label and was irreversibly incorporated mainly in the α subunit. We then found that all subunits were labelled depending on the ligand/receptor concentration ratio. The Scatchard analysis of saturation curves (figure 1) obtained with the purified receptor showed a K_d value of $(4.8 \pm 1.9) \mu\text{M}$ and a B_{max} of $(1.5 \pm 0.3) \mu\text{M}$ ($n = 3$). Preliminary experiments, indicated an IC_{50} value of 4 mM and a K_i of $(1.6 \pm 0.3) \mu\text{M}$ ($n = 2$). The effect of carbamylcholine on the ^3H -5HT binding was studied. Competition experiments and autoradiographies suggested a competitive interaction in the millimolar range. The above results indicate that the nAChR possesses a low affinity site for serotonin. Further studies are being performed in order to better characterize such interaction.

References

- Cross KML, Foreman RC, Chad JE (1995) Enhancement by 5-hydroxytryptamine and analogues of desensitization of neuronal and muscle nicotinic receptors expressed in *Xenopus* oocytes. *British Journal of Pharmacology* 114:1636–1640.
- García-Colunga J, Mileti R (1996) Serotonergic modulation of muscle acetylcholine receptors of different subunit composition. *PNAS USA*, 93:3990–3994.
- Otero de Bengtsson MS, Biscoglio de Jiménez Bonino MJ (1997) Hydroxytryptamine as a modulator of the nicotinic acetylcholine receptor. *J. Neurochem.*, 69:Supp, S76B.
- Palma E, Mileti AM, Eusebi F, Mileti R (1996) Neuronal nicotinic threonine-for-leucine 247 $\alpha 7$ mutant receptors show different gating kinetics when activated by acetylcholine or by the noncompetitive agonist 5-hydroxytryptamine. *PNAS USA* 93: 11231–11235.

82

Microinjection of cultured sympathetic neurons: A method for studying the regulation of gene transcription

F. Pajak, L. Houhou, M.J. Lecomte, M. Fauquet, J. Mallet, S. Berrard

CNRS, Laboratoire de Génétique de la Neurotransmission et des Processus Neurodégénératifs, Hôpital Pitié-Salpêtrière, 75013 Paris, France

Primary cultures of sympathetic neurons from superior cervical ganglia (SCG) of newborn rats constitute an attractive model system to investigate the mechanisms that govern the acquisition of the cholinergic phenotype. These neurons, initially noradrenergic, can become cholinergic in response to different extracellular factors, such as the cytokine CDF/LIF and retinoic acid (RA). In particular, these factors decrease both the activity and mRNA levels of enzymes involved in the synthesis of noradrenaline, tyrosine hydroxylase (TH) and dopamine β -hydroxylase (DBH). Simultaneously, they increase the activity of choline acetyltransferase (ChAT) and the number of molecules of the vesicular transporter of acetylcholine (VACHT), as well as the amount of ChAT and VACHT mRNA. These data suggest that CDF/LIF and RA may up-regulate the transcription of the ChAT and VACHT genes, which are organized as embedded genes, and concomitantly down-regulate the transcription of the genes encoding TH and DBH.

To study the mechanisms that regulate the expression of these genes, cultured SCG neurons need to be transiently transfected with reporter plasmids containing the promoter of the gene of interest. Classical methods (electroporation, calcium phosphate precipitation, lipofection, polyethylenimine-mediated transfection) were ineffective to transfect these neurons. Several studies have reported the use of microinjection to transfer genes into primary cultured neurons, but without any quantification of the expression of these genes (Garcia et al., 1992; Gagliardini et al., 1994; Chireux et al., 1994). In this study, we exploited the technique of microinjection to deliver plasmids into SCG neurons and to obtain quantifiable and reproducible reporter gene activities.

Neurons were grown on glass coverslips and about 1000 molecules of plasmid were injected into the nuclei of neurons. Injection of plasmids with reporter genes encoding β -galactosidase or the green fluorescent protein revealed a good survival of the injected neurons. A plasmid containing the gene of firefly luciferase under the control of the promoter sequences of the Rous sarcoma virus (RSV) was then injected in 100 neurons and a very high luciferase activity (more than 105 that of the assay background) could be detected 24 h after injection. The sensitivity of this technique allows the detection of activity in a single injected neuron. To assess in-

jection efficiencies, a second plasmid was co-injected, in which a constitutively active promoter directs the expression of the Renilla luciferase gene. The activity of this latter luciferase is measured in the same cell homogenate as that used for the former, and is used to normalize it. In these conditions, the quantitative determination of the reporter gene activity was reproducible.

We then tested if a CDF/LIF responsive promoter is still regulated when microinjected in SCG neurons. CDF/LIF treated and untreated neurons were injected with a plasmid containing three cytokine-response elements of the peripherin gene inserted upstream from the thymidine kinase promoter linked to the firefly luciferase gene (Lecomte et al., 1998). A higher normalized reporter gene activity was detected in treated neurons, revealing that the up-regulation of a gene transcription by CDF/LIF can be measured in microinjected neurons.

A plasmid, in which about 5.2 kb of the rat VACHT gene promoter was linked to firefly luciferase gene, was then injected in the neurons. A high level of activity was detected after 24 h of expression, indicating that the activity of the VACHT gene promoters can be quantified using this method.

Taken together, these results reveal that microinjection of plasmid DNA is well suited to study gene regulation in cultured sympathetic neurons, in particular that of the ChAT and VACHT genes.

References

- Chireux M, Raynal JF, Le Van Thai A, Cadas H, Bernard C, Martinou I, Martinou JC, Weber MJ (1994) Multiple promoters of human choline acetyltransferase and aromatic L-amino acid decarboxylase genes. *J. Physiol.* 88, 215–227.
- Gagliardini V, Fernandez PA, Lee RKK, Drexler HCA, Rotello RJ, Fishman MC, Yuan J (1994) Prevention of vertebrate neuronal death by the *crmA* gene. *Science* 263, 826–828.
- Garcia I, Martinou I, Tsujimoto Y, Martinou JC (1992) Prevention of programmed cell death of sympathetic neurons by the bcl-2 proto-oncogene. *Science* 258, 302–304.
- Lecomte MJ, Basseville M, Landon F, Karpov V, Fauquet M (1998) Transcriptional activation of the mouse peripherin gene by leukemia inhibitory factor: involvement of the STAT proteins. *J. Neurochem.* 70, 971–982.

Kinetic and thermodynamic effects of acetylcholinesterase on the solubility of A β peptide

D. Pérez, R. Alarcón, N.C. Inestrosa

Unidad de Neurobiología Molecular, Departamento de Biología Celular y Molecular, P. Universidad Católica de Chile, Chile

The principal hallmark of Alzheimer's disease is the presence of extracellular senile plaques, which consist of deposits of amyloid filaments. The main component of these compacted deposits is the amyloid- β -peptide (A β) of 40-42 amino acids, besides which the senile plaques also contain other proteins such as acetylcholinesterase (AChE). This enzyme directly promotes the assembly of A β peptide into amyloid fibrils (Inestrosa et al., 1996). The peptide has a critical concentration (Cr) below which it does not aggregates (thermodynamic solubility); over Cr, the A β peptide requires the formation of nuclei to trigger fibrillogenesis, thus determining the kinetic solubility of the peptide (Lansbury, 1997). However, neither the mechanism by which AChE promotes A β fibrillogenesis, nor the stage at which the enzyme exerts its effect, are known.

In this work, we dissect the effect of AChE on fibrillogenesis between its kinetic and thermodynamic components. In multi-point kinetic studies using turbidity, we observed a two-fold reduction in the lag time of amyloid fibril elongation when the enzyme was present in a molar ratio of 1:250 (figure 1), two molecular forms of AChE was used, the amphiphilic G4 and the hydrophilic G1, we observed the same reduction in lag period with both of them. Once the system reached an equilibrium state, measured as a constant turbidity value, we determined the Cr of the A β aggregation process by two independent methods: densitometric analysis of SDS-PAGE and fluorimetric analysis. In the latter, extrapolation to zero of maximal fluorophor emission values, obtained using different A β concentrations, allowed us to estimate a Cr of 44.4 ± 2.8 mM. A similar determination of Cr was obtained in presence of varying AChE concentrations (10–100 nM), this results are summarized in table I. The solubility of A β was seen to decrease as the AChE concentration increased. Next, we calculated the apparent association constant for A β polymerization (K_{app}) using the relation $K_{app} = Cr^{-1}$ (Lee et al., 1977). The analysis of the association constants allow us to determine the true Cr for the A β peptide in presence of AChE, obtained a value of 24.04 ± 0.8 mM. Both aforementioned analytical methods showed the same decrease in A β solubility: 1.8-fold. Next we applied the Wyman analysis plotting $\ln K_{app}$ v/s $\ln [AChE]$ (Lee et al., 1977); the slope of this plot revealed that the effect of AChE on the solubility of A β is due to the preferen-

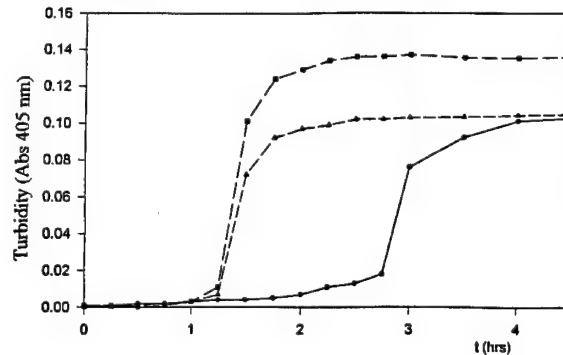


Figure 1. Turbidity of A β peptide aggregation. The assay was carried out at 75 μ M of A β alone (circles) or in presence of AChE G1 (squares) or AChE G4 (triangles) at 1: 250 MR (respect the catalytic subunits).

tial binding of AChE to the polymeric form of the peptide rather than its monomeric state. Secondly, we were able to establish a stoichiometry of one molecule of AChE for every two molecules of A β peptide incorporated into the growing fibril. Finally, our results suggest that AChE promotes the A β aggregation by acting both as an heterologous seed (kinetic effect) and by reducing the solubility of the peptide (thermodynamic effect). This work was supported by a grant from FONDECYT N°1971240 and by a Presidential Chair in Science from the Chilean Government to NCI.

References

- Inestrosa NC, Alvarez A, Perez CA, Moreno RD, Vicente M, Linker C, Casanueva OI, Soto C, Garrido J (1996) Acetylcholinesterase accelerates assembly of amyloid -beta-peptide into Alzheimer's fibrils: possible role of the peripheral site of the enzyme. *Neuron* 16, 881–891.
- Lansbury PT Jr. (1997) Structural neurology: are seed at the root of neuronal degeneration? *Neuron* 19, 1151–1154.
- Lee JC, and Timashef SN (1977) In vitro reconstitution of calf brain microtubules: effect of solution variables. *Biochemistry* 16, 1754–1764.

Table I. Dependence of A β -amyloid solubility parameters on AChE concentration. The results are means \pm S.D., $n = 3$, $P < 0.05$.

[AChE] Total (nM)	Ccr (μ M)	K _{app} (L/mol)	G ^{app} (kcal/mol)
0	44.4 \pm 2.8	2.26 \pm 0.14 10^4	5.94 \pm 0.04
10	40.7 \pm 1.4	2.46 \pm 0.08 10^4	5.99 \pm 0.02
25	31.0 \pm 1.1	3.23 \pm 0.11 10^4	6.15 \pm 0.02
50	23.7 \pm 1.4	4.23 \pm 0.25 10^4	6.31 \pm 0.04
100	18.9 \pm 3.5	5.42 \pm 0.89 10^4	6.46 \pm 0.11

$$\Delta G^{\text{app}} = -RT \ln K_{app}$$

84

SPOCK, a novel proteoglycan in a cholinergic synapse: The vertebrate neuromuscular junction

J.-P. Périn, C. Cifuentes-Diaz, F. Charbonnier, D. Goudou, F. Rieger, P.M. Alliel

Laboratoire de Neuromodulations Interactives et Neuropathologies, INSERM, 17, rue du Fer-à-Moulin, 75005 Paris, France

SPOCK (Charbonnier et al., 1998), also known as 'testican' (Alliel et al., 1993), is a modular proteoglycan. SPOCK is the acronym of the peptidic modules defined within the proteoglycan's protein core. It designates motifs related to SPARC/Osteonectin and also depicts a 46 amino acid long domain centered around a CWCV (cysteine-tryptophan-cysteine-valine) tetrapeptide, and a Kazal-like protease inhibitory polypeptide, also present in the acetylcholine receptor (AChRs) clustering molecule agrin (Patty and Nikolics 1993), which has been characterized as a proteoglycan (Tsen et al., 1995). In addition to these structural features, SPOCK has been shown to be highly expressed in a subset of synapses within the mouse brain, in a postsynaptic location (Bonnet et al., 1996). These observations, and data dealing with the involvement of proteoglycans in AChRs clustering at the neuromuscular junction (NMJ) (Ferns et al. 1993), prompted us to evaluate the occurrence of SPOCK at the NMJ.

The present report deals with the immunohistochemical detection of SPOCK in normal murine skeletal muscle with the concomitant detection of AChRs. This study was carried out with antibodies raised in rabbits (719-7 and PTC9), directed against two chimeric proteins expressed in bacterial systems and corresponding to the SPOCK N- and C-terminal regions respectively (figure 1).

SPOCK is found in the basal lamina of the muscle fibers. It appears highly concentrated at the NMJ and partially colocalized with the AChRs revealed by an α -bungarotoxin staining. A positive signal is also observed in interstitial nerves. Both antibodies gave rise to the same signals. Figure 2 presents the SPOCK expression pattern obtained with the 719-7 immunoserum. The reaction was specifically inhibited by preincubation of the 719-7 immunoserum with increasing amounts of the corresponding antigen.

The detection of SPOCK in intramuscular nervous branches led us to examine its distribution in peripheral isolated nerves. A strong signal was observed in Schwann cells, in axons, and at the node of Ranvier.

Thus, SPOCK is a new molecule, belonging to the expanding proteoglycan family, whose structural features and expression pat-

terns are indicative of an involvement in the synaptic architecture at the NMJ. Experiments, currently in progress, to delineate its expression during development, are in agreement with a possible function of SPOCK in synapse formation or stabilization.

References

- Alliel, P.M., Périn, J.-P., Jolles, P. & Bonnet, F. (1993). Testican, a multidomain proteoglycan resembling modulators of cell social behaviour. *Eur. J. Biochem.* 214, 347-350

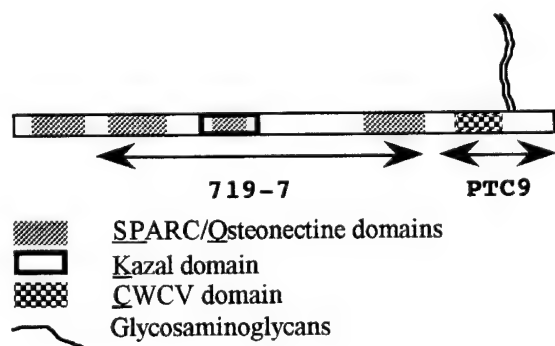


Figure 1. Localization, within the SPOCK protein core, of the expressed polypeptides used for the 719-7 and PTC-9 immunera production. The modules are boxed.

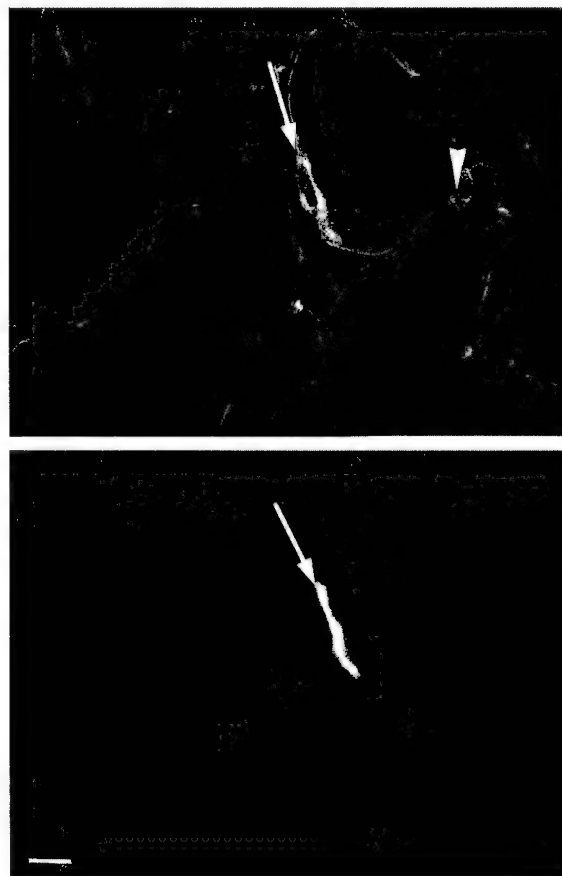


Figure 2. SPOCK expression at the neuromuscular junction. Transverse sections of adult mouse gastrocnemius muscle were double stained with the anti-recombinant SPOCK antibody 719-7 (top) and α -bungarotoxin (bottom). Immunoreactivity was present at the synaptic sites (arrows) identified by α -bungarotoxin binding as well as in interstitial nerve fibres (arrowheads). Scale bar 25 μ m.

Bonnet, F., Périn, J.-P., Charbonnier, F., Camuzat, A., Roussel, G., Nussbaum, J.-L. & Alliel, P.M. (1996). Structure and cellular distribution of mouse brain testican. Association with the postsynaptic area of hippocampus pyramidal cells. *J. Biol. Chem.* 270, 3392–3399.

Charbonnier, F., Périn, J.-P., Mattei M.G., Camuzat A., Gressin L. & Alliel P.M. (1998). Genomic organization of the human SPOCK gene and its chromosomal localization to 5q-31. *Genomics* 48, 377–380.

Ferns, M.J., Campanelli, J.T., Hoch, W. & Hall, Z. (1993). The ability of agrin to cluster AChRs depends on alternative splicing and on cell surface proteoglycans. *Neuron* 11, 491–502.

Patty, L. & Nikolics, K. (1993). Functions of agrin and agrin related proteins. *Trends Neurosci.* 16, 76–81.

Tsen, G., Halfter, W., Kröger, S. & Cole G.J. (1995). Agrin is a heparan sulfate proteoglycan. *J. Biol. Chem.* 270, 3392–3399.

85

Opposite cerebrovascular responses to physostigmine and tacrine in the cortex deafferented from the nucleus basalis magnocellularis suggest direct projections from this nucleus to intracortical inhibitory neurons and microvasculature

P. Peruzzi, D. von Euw, J. Seylaz, P. Lacombe

Laboratoire de Recherches Cérébrovasculaires, CNRS-UPR 646, Université Paris 7, IFR 6, Faculté Lariboisière-Saint-Louis, 10, avenue de Verdun, 75010 Paris, France

Cholinesterase inhibitors used to treat Alzheimer's disease are thought to compensate for the functional impairment of the cholinergic basalocortical system. However, previous results obtained by measuring CBF in rats shortly after lesion of the nucleus basalis magnocellularis (NBM) did not support this contention (1, 2). In this study we investigated the cerebrovascular responses to physostigmine and tacrine late after the lesion. We sought to determine whether denervation supersensitivity occurred in the projection areas of the impaired NBM and enhanced the responsiveness to these cholinesterase inhibitors.

Regional CBF was measured using the [14 C]iodoantipyrine tissue sampling technique in conscious rats i.v. infused with physostigmine (0.1 or 0.2 mg/kg/h), tacrine (3 or 8 mg/kg/h), or saline, 3–5 weeks after unilateral lesioning of the NBM with ibotenic acid.

Physostigmine and tacrine dose-dependently increased blood flow in most cortical and subcortical regions compared to the control group. Unexpectedly, late after the lesion, interhemispheric comparisons showed that physostigmine caused dose-related smaller increases in blood flow in most cortical areas of the deafferented hemisphere. In contrast, tacrine caused greater blood flow increases in the frontal cortex of the lesioned hemisphere.

The results suggest that both physostigmine and tacrine stimulate a 'vasodilator' intracortical neuron and simultaneously another target responsible for the NBM-dependent component of their cerebrovascular effects. This component is of opposite nature according to the agonists used and presumably involves different postsynaptic targets. A reduced responsiveness to physostigmine occurring at 3–5 weeks postlesion can be explained by denerva-

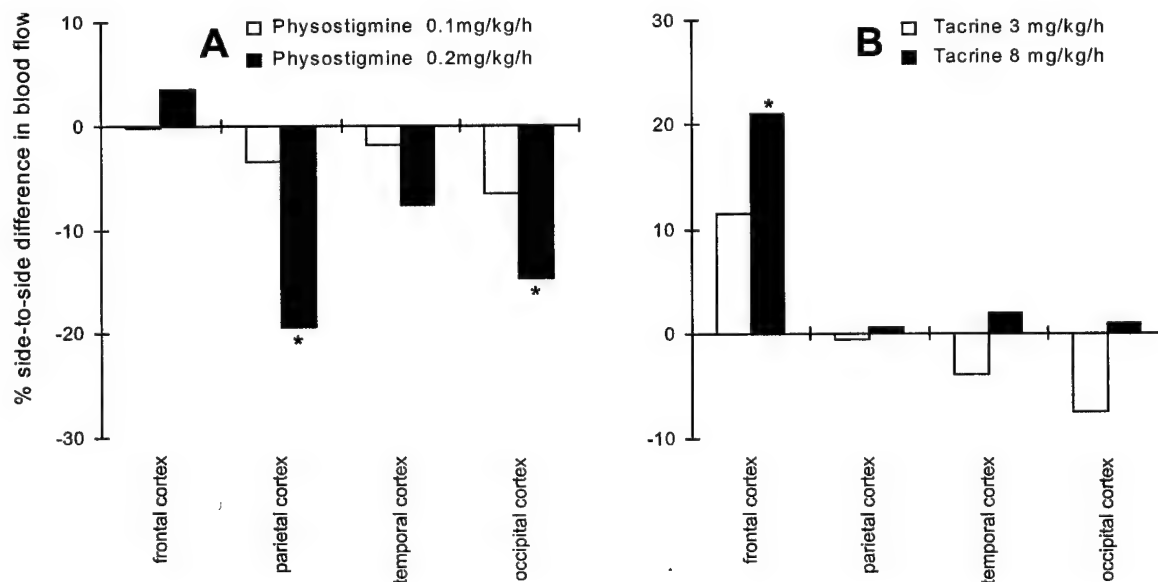


Figure 1. Cortical distribution of the side-to-side differences (with respect to the intact side) in blood flow in response to physostigmine (A) and tacrine (B), 3–5 weeks after unilateral NBM lesioning. The effects display the NBM-dependent component of the responses. Physostigmine induced dose-dependent smaller blood flow responses on the lesioned side, with the notable exception of the frontal cortex. In contrast, tacrine induced a dose-dependent greater blood flow response in the deafferented frontal cortex. *Significantly different from the intact side ($P < 0.05$, paired t -test).

tion supersensitivity of a synapse of NBM projections on to a cortical inhibitory interneuron. The facilitated increase in perfusion in response to tacrine in the deafferented hemisphere can probably be ascribed to a non-neuronal NBM target. This tacrine-specific effect occurs in the first week after the lesion (2) and then remains stable as long as 5 weeks after the lesion. It may involved the neurovascular junction of NBM terminals in the microvessel environment.

Altogether, we suggest that the cholin-sensitive 'vasodilator' neuron of the cortical circuitry stimulated by physostigmine and tacrine is connected to an inhibitory interneuron. The latter could receive direct NBM projections and be brought into play mostly by physostigmine. The tacrine-specific effect could rely on a direct influence of NBM projections on the cortical microvasculature, which includes perivascular astrocytes (3).

Hence, physostigmine, tacrine, and perhaps all cholinesterase inhibitors, do not properly fulfill the principle of cholinergic replacement therapy for Alzheimer's disease, as they cannot compensate for a deficit of NBM functioning. However, the additional property of tacrine is beneficial to blood flow in a cortical area deafferented from the NBM. This tacrine-specific effect refers to brain perfusion in Alzheimer's disease. Cortical blood flow is highly sensitive to NBM activation (4), an effect which is doubled under physostigmine (5) and halved in aged rats (6). Consistent with studies showing that brain microvessels are the site of pathophysiological changes in Alzheimer's disease (6), our results support the idea that the clinical improvement obtained by pro-

longed tacrine treatment could be ascribed to such a circulatory effect. Moreover, the cerebrovascular reactivity to acutely administered tacrine might be a valuable index of its therapeutic efficacy.

References

- 1 Peruzzi P, Lacombe P, Moro V, Vaucher E, Levy F, Seylaz J. The cerebrovascular effects of physostigmine are not mediated through the substantia innominata. *Exp. Neurol.* 122: 319-326, 1993.
- 2 Peruzzi P, Borredon J, Seylaz J, Lacombe P. Tacrine overcompensates for the decreased blood flow induced by basal forebrain lesion in the rat. *NeuroReport* 8: 103-108, 1996.
- 3 Kacem K, Lacombe P, Seylaz J, Bonvento G. Structural organization of the perivascular astrocyte endfeet and their relationship with the endothelial glucose transporter: a confocal microscopy study. *Glia* 23: 1-10, 1998.
- 4 Vaucher E, Borredon J, Bonvento G, Seylaz J, Lacombe P. Autoradiographic evidence for flow-metabolism uncoupling during stimulation of the nucleus basalis of Meynert in the conscious rat. *J. Cereb. Blood Flow Metab.* 17: 686-694, 1997.
- 5 Dauphin F, Lacombe P, Sercombe R, Hamel E, Seylaz J. Hypercapnia and stimulation of the substantia innominata increase rat frontal cortical blood flow by different cholinergic mechanisms. *Brain Res.* 553: 75-83, 1991.
- 6 Sercombe R, Lacombe P, Verrecchia C, Springhetti V, MacKenzie ET, Seylaz J. Basal forebrain control of cortical blood flow and tissue gases in conscious aged rat. *Brain Res.* 662: 155-164, 1994.
- 7 Kalara RN. Cerebral vessels in ageing and Alzheimer's disease. *Pharmacol. Ther.* 72: 193-214, 1996.

Some theoretical aspects of the allosteric interactions at muscarinic receptors

J. Proška^{a, b}, M. Urbánský^b

^aVUFB, a.s., Research Institute for Pharmacy and Biochemistry, Kourimska 17, 130 60 Prague, Czech Republic

^bInstitute of Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 160 00 Prague, Czech Republic

Gallamine, the first allosteric ligand to be examined in detail, decreases the binding of muscarinic antagonist N-methylscopolamine (NMS) (Stockton et al., 1983). In contrast, alcuronium, another neuromuscular blocking agent, increases the binding of NMS (Tucek et al., 1990; Proška and Tucek, 1994).

Both above-mentioned allosteric ligands slow down dramatically the association and dissociation kinetics of NMS when it binds to muscarinic receptors. One possible interpretation is that these allosteric ligands either in a direct or indirect manner entirely prevent NMS dissociation and association (Proška and Tucek, 1994; Matsui et al., 1995).

Progress in the field is hampered by the unavailability of radiolabeled allosteric ligands with an affinity high enough to be used in the binding assay. The published methods of detection and quantitation of allosteric interactions at muscarinic receptors (Lazareno and Birdsall, 1995; Ellis and Seidenberg, 1992) postulate that allosteric ligand has fast kinetics, relative to those of NMS. The semiempirical methods cannot afford to estimate, by definition, the values of the allosteric ligand dissociation and association rate constants.

We have synthesized two apparently purely allosteric ligands: anatruxonium (we repeated the synthesis of it (Kharkevich and Skoldinov)) and URA 114 (Urbánský and Proška, 1997) are negative and positive respectively allosteric modulators of [³H]NMS

binding to M₂ receptors. The magnitudes of their affinities were found to be comparable with those of [³H]NMS. We have analysed, in detail, the effects of increasing concentrations of the allosteric ligands on the time course of [³H]NMS binding. Our results were consistent with a ternary complex theory. The data were analyzed with Kinsim (Frieden, 1993), and Mathematica 3.0.1. (Wolfram Research Inc., 1997) software packages. We discuss the crucial difference between the kinetic behaviour of the systems described by the circular four-membered scheme or four-membered linear scheme. Supported by grant 203/97/0302 from the Grant Agency of the Czech Republic and from personal source (J.P.).

References

- 1 Ellis J, Seidenberg M (1992) Two allosteric modulators interact at a common site on cardiac muscarinic receptors. *Mol. Pharmacol.* 42: 638-641
- 2 Frieden C (1993) Numerical integration of rate equations by computer. *Trends Biochem. Sci.* 18: 58-60
- 3 Kharkevich DA, Skoldinov AP (1986) The derivatives of carboxylic acids, in *New Neuromuscular Blocking Agents: Handbook of Experimental Pharmacology*, vol. 79 (D.A. Kharkevich, ed.). Springer Verlag, Berlin, 323-369
- 4 Lazareno S, Birdsall NJM (1995) Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of

- strychnine and acetylcholine at muscarinic receptors. *Mol. Pharmacol.* 48, 362–378
- Mathematica 3.0.1. (1997) Wolfram Research Inc., Champagne, IL
- Matsui H, Lazareno S, Birdsall NJM (1995) Probing of the location of the allosteric site on m1 muscarinic receptors by site-directed mutagenesis. *Mol. Pharmacol.* 47, 88–98
- Proska J, Tucek S (1994) Mechanisms of steric and cooperative actions of alcuronium on cardiac muscarinic acetylcholine receptors. *Mol. Pharmacol.* 44, 709–717
- Stockton JM, Birdsall NJM, Burgen ASV, Hulme EC (1983) Modification of the binding properties of muscarinic receptors by galamine. *Mol. Pharmacol.* 23, 551–557
- Tucek S, Musilkova J, Nedoma J, Proska J, Shelkovnikov S, Vorlicek J Positive cooperativity in the binding of alcuronium and N-methylscopolamine to muscarinic receptors. *Mol. Pharmacol.* 38, 674–680
- Urbansky M, Proska J (1997) Truxillic acid derivatives: High affinity, M2 selective allosteric modulators. Probes for mapping the muscarinic receptors. *Life Sci.* 60, 1170

87

Constitutive inhibitory action of muscarinic receptors on adenylate cyclase in cardiac membranes: effects of atropine, S-(–)-hyoscyamine, and R-(+)-hyoscyamine

J. Rícný^a, F. Gualtieri^b, S. Tucek^a

^aInstitute of Physiology, Academy of Sciences of the Czech Republic, Videnská 1083, 14220 Prague, Czech Republic

^bDepartment of Pharmaceutical Sciences, University of Florence, Via G. Capponi 9, 50121 Florence, Italy

Muscarinic receptors in the heart have been shown to display agonist-independent spontaneous (constitutive) activity which causes changes in the opening of cardiac ion channels and in the activity of G proteins. We investigated whether an inhibition of the constitutive activity of muscarinic receptors induced by the binding of their antagonists brings about a change in the synthesis of cyclic AMP in rat cardiac membranes. Atropine and S-(–)-hyoscyamine were indeed found to enhance the forskolin-stimulated synthesis of

cyclic AMP in rat ventricular membranes by up to 24%. The effect was stereospecific and the potency of R-(+)-hyoscyamine was more than 20-fold lower than that of the S-(–) enantiomer, confirming that the action of hyoscyamine is receptor-mediated. The effect did not depend on the presence of endogenous acetylcholine in the system used. The results suggest that the adenylate cyclase in the heart is exposed to continuous mild inhibition by the constitutively active muscarinic receptors in the membranes of cardiomyocytes.

88

Changes in gene expression in basal ganglia and associated structures following local fasciculin application in the striatum and in the substantia nigra

M. Rogard, M.J. Besson

Institut des Neurosciences, laboratoire de Neurochimie Anatomie, UMR 7624, 9, quai Saint-Bernard 75005 Paris, France

The basal ganglia are structures involved in co-ordination of movements. There, acetylcholine (ACh) plays a critical role in the physiology of these structures. In the striatum ACh is synthesised in cholinergic interneurons which correspond to about 2% of the total neuronal population. Although a limited number of striatal neurons are able to synthesise ACh and its degradation enzyme, acetylcholinesterase (AChE), a high concentration of AChE is found in the striatum. In the substantia nigra (SN), AChE is also present and is synthesised in dopaminergic neurons. In both structures AChE can be released consecutively to changes in neuronal activity (Greenfield et al., 1980, 1983).

To examine potential roles played by AChE we analysed in the striatum and the SN the effects produced by a prolonged inhibition of AChE activity on striatal and nigral gene expression (peptides, enzymes and immediate early genes). This was investigated by locally applying fasciculin (10^{-5} M, 4 μ L), a snake venom known to irreversibly block AChE activity (Karlsson et al., 1984).

In the striatum, AChE activity was inhibited by 80% for at least 72 h after fasciculin application and normal activity recovered in 1 week. At 24 h, the binding of 3 H-pirenzepine (a ligand which labels m1 muscarinic receptors) was reduced by 25% in-

dicating that the toxin binds to this receptor subtype by mimicking some properties of the m1 toxin (Max et al., 1993). Although AChE activity was markedly inhibited, AChE mRNA expression in striatal cholinergic interneurons remained unchanged at 24 h. In striatal efferent neurons which are under a cholinergic control, the expression of glutamic acid decarboxylase (GAD67) and substance P mRNA expression remains unchanged in striato-nigral neurons. In striato-pallidal neurons, GAD67 mRNA expression remained unchanged but preproenkephalin (PPE) mRNA expression was slightly increased. Moreover, at distance, a marked increase in PPE mRNA expression was observed in the piriform cortex (see figure 1) ipsilateral to the injection suggesting the activation of a polysynaptic striato-cortical loop following striatal AChE inhibition.

In the SN, AChE activity was less affected by fasciculin since it was inhibited by 45% and 20% at 24 and 72 h, respectively, after fasciculin application. The differential toxin efficacy to inhibit AChE activity in the striatum and the SN could be attributed to a differential localisation (extra- versus intracellular) of AChE in these two structures. In the SN, the expression of AChE and tyrosine hydroxylase mRNAs was inhibited when fasciculin was

injected in the pars compacta where are localised dopaminergic neurons. Furthermore, the expression of the immediate early gene Zif 268 which has been used to visualise activated neurons (Sukhatme et al., 1988; Simonato et al., 1991) was examined following nigral fasciculin application. Whereas no local effect was observed, zif 268 mRNA levels were markedly increased at distance in the dentate gyrus (granular cells) and in the CA1 to CA4 regions (pyramidal cells) of the hippocampus. Of interest, the organo-phosphorus-induced convulsions could involve activation of such polysynaptic pathways.

In conclusion, irreversible inhibition of striatal and nigral AChE can modify locally the neuronal activity as evidenced by changes in peptide or enzyme expression as well as at distance as evidenced by distal changes in peptide and immediate early gene expression. Work supported by grant no 25-179/DRET/966ETCA/CEB/B.

References

- Greenfield, S.A., Cheramy, A., Leviel, V., Glowinski, J. (1980) In vivo release of AChE in the cat substantia nigra and caudate nucleus. *Nature*, 284, 355-357.
- Greenfield, S.A., Grunewald, R.A., Foley, P., Shaw, S.G. (1983) Origin of various enzymes released from substantia nigra and caudate nucleus: effect of 6-OHDA lesions of the nigro-striatal pathway. *J. Comp. Neurol.*, 214, 87-92.
- Karlsson, E., Mrugua, P.M. and Rodriguez-Ithurralde, D. (1984) Fasciculin, anticholinesterase toxins from the venom of the green mamba *Dendroaspis angusticeps*. *J. Physiol.* 79, 232-240.
- Max, S.I., Liang, J.S. and Potter, L.T. (1993). Purification and properties of ml-toxin, a specific antagonist of m1 muscarinic receptors. *J. Neurosci.* 13, 4293-4300.
- Simonato M., Hosford DA., Labiner DM., Shin C., Mansbach HH., McNamara JO (1991) Differential expression of immediate early genes in the hippocampus in the kindling model of epilepsy. *Mol. Brain Res.* 11, 115-124.

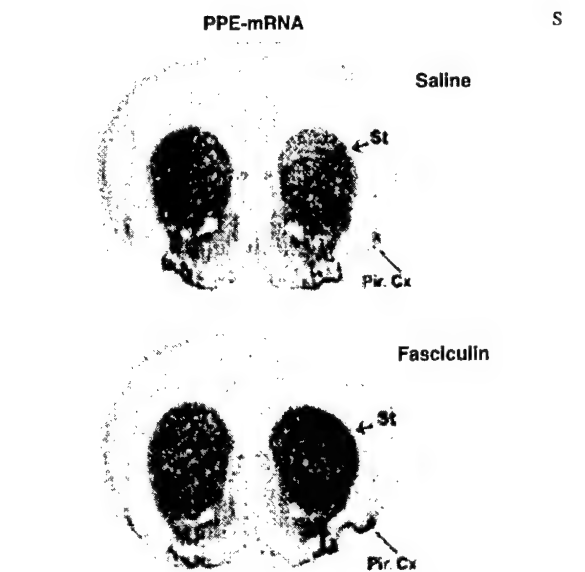


Figure 1. The expression of PPE mRNA was examined by in situ hybridisation 24 h after the injection of saline (upper part) or fasciculin (lower part) in the right striatum (St, short arrow). In fasciculin-treated rats, PPE mRNA levels were increased locally in the injected striatum and distally in the piriform cortex (Pir. Cx) ipsilateral to the fasciculin injection.

ukhatme V.P., Cao X.M., Chang L.C., Tsai-Morris C.H., Stamenkovich D., Ferreira P.C., Cohen D.R., Edwards S.A., Shows T.B. and Curran T. (1988). A zinc finger-encoding gene coregulated with c-fos during differentiation, and after cellular depolarization. *Cell* 53, 37-43.

Cholinergic mechanisms in plant fertilization

V.V. Roshchina

Russian Academy of Sciences Institute of Cell Biophysics, 142292 Pushchino, Moscow Region, Russia

Cholinergic mechanisms appear to exist in every living cell, not only belonging to vertebrates. Acetylcholine (ACh) and biogenic amines, neuromediators also found in plant cells, can play role of both signalling substances and growth regulators (Roshchina, 1991) ACh and the activity of cholinesterase was observed in the flowers: the pistil stigma of different species (Roshchina and Semenova, 1995) and in exine of pollen grains (Roshchina et al., 1994). To show a possible role of cholinergic mechanisms in a regulation of a fertilisation is the aim of the study. We analyzed: 1) the effects of ACh and its antagonists on the fertilization, in particular pollen germination in vivo and in vitro, as well as autofluorescence of pollen and pistil; 2) the cholinesterase activity in the reproductive organs during pollination and fertilization as well as the contribution of cholinesterase to the processes; 3) the action of cholinesterase inhibitors on the fruit and seed formation after flower organ treatment.

Objects of the study were pollen and pistils of *Hippeastrum hybridum* and of three clones of *Petunia hybrida* differing in functional activity of male gametophyte (self-compatible clone, self-

incompatible clone and sterile clone) grown in greenhouse were investigated. Pollen and pistil stigma autofluorescence under 360-380 nm light excitation was studied (Roshchina and Melnikova, 1996). Pollen germination in vitro was estimated as the pollen tube formation on the artificial nutrient medium, including 10% sucrose (Roshchina and Melnikova, 1996) whereas the process in vivo was seen as the fruit and seed formation. The fruits and seeds are produced after both self- and crosspollination in *Hippeastrum hybridum* and *Petunia hybrida* of self-compatible clone whereas in *Petunia hybrida* of the self-incompatible clone (a male gametophyte inhibition) it occurs only after cross-pollination, and in sterile clone (male sterility), the seeds are also formed only after cross-pollination. The cholinesterase activity was measured in extracts from pollen or pistils (Roshchina et al., 1994). After a month, the seed production was estimated.

As for *Hippeastrum*, ACh and their antagonists had effects on the pistil stigma autofluorescence, pollen germination in vitro and seed formation after the pistil treatment (table 1), whereas the pollen grain autofluorescence was insensitive to them.

Table I. The effect of anticholinergic drugs, applied to the pistil stigma or pollen, on the formation of seeds of *Hippeastrum hybridum*. S, stimulation; NE, no effect; NS, no stimulation.

Substance	Autofluorescence of pistil	Pollen germination in vitro	Seed formation
ACH 10^{-5} M	S	S	Normal
Atropine 10^{-5} M	NE	NE	Absent
d-Tubocurarine 10^{-5} M	NE	NE	Absent
Atropine 10^{-5} M + ACH 10^{-4} M	NS	NS	Absent
d-Tubocurarine 10^{-5} M + ACH 10^{-4} M	NS	S	Absent
Neostigmine 10^{-5} M	S	S	S
Physostigmine 10^{-5} M	S	S	NE

ACH stimulates the fluorescence intensity of the stigma approximately two-fold, whereas after the preliminary treatment by d-tubocurarine or atropine the effects are not observed. It shows the receptors of acetylcholine exist on the stigma surface. The ACH effects on pollen germination depend on the initial control level. When the index of germination is low acetylcholine stimulates the process ~two-fold above the control. Whereas, at high control germination rates there is little effect. Atropine also stimulated the pollen germination. When ACH was added after the treatment by d-tubocurarine, it is more stimulated than after the antagonist alone, in contrast to atropine, blocker of muscarinic type of animal cholinergic receptor. The sensitivity of a pistil stigma to ACH and their antagonists may be studied in vivo as a fertilization (treatment with anticholinesterase drugs, neostigmine and physostigmine, and with blocker of nicotinic cholinergic receptor, alkaloid d-tubocurarine. Moreover, the visible seed formation is seen later than in a control. In the case of tubocurarine and atropine, the seeds did not mature).

The hydrolysis of acetylcholine was observed in the extracts from both pollen and pistils (table II). The participation of cholinesterase was evidenced from the significant decrease in rate of acetylthiocholine degradation by the inhibitors of cholinesterase, physostigmine and neostigmine (table II). In the extracts from petunia pollen, the rate of choline ester hydrolysis was not inhibited by high substrate concentrations. The reaction rate was 3–4 times higher with acetylcholine than with butyrylthiocholine. The Michaelis constants (K_m) were 6×10^{-4} M with acetylthio-

choline and 7×10^{-4} M with butyrylthiocholine, respectively, for the enzymatic reaction inhibited by heating at 70 °C. High rate of acetylthiocholine hydrolysis in the pollen of two fertile clones was suppressed by 90% with the inhibitors of cholinesterase (table II). Male sterility correlates with the cholinesterase lack or decrease of the enzyme activity. All the phenomena suggest the participation of cholinergic receptor and cholinesterase in fertilization. The study was supported by a grant of the Russian Fund for Fundamental Studies N 96-04-48091.

References

- Roshchina VV (1991) Biomediators in Plants. Acetylcholine and Biogenic Amines. Biological Center of USSR Academy of Sciences, Pushchino, 192 pp.
- Roshchina VV, Melnikova EV, Kovaleva LV, Spiridonov NA (1994) Cholinesterase in pollen grains. Doklady. Biological Sciences 337, 424–427
- Roshchina VV, Semenova MN (1991) Plant cholinesterases: activity and substrateinhibitory specificity. J. Evol. Biochem. Physiol. 26, 644–651
- Roshchina VV, Melnikova EV (1996) Microspectrofluorimetry: A new technique to study pollen allelopathy. Allelopathy Journal. 3, 51–58.
- Roshchina VV, Semenova MN (1995) Neurotransmitter systems in plants. Cholinesterase in excreta from flowers and secretory cells of vegetative organs in some species. Proceedings of the Plant Growth Regulation Society of America, (Eds. D.Greene and G.Cutler) 22 Annual Meeting, July18–20, 1995, Minneapolis: Fritz C.D. pp.353–357.

Table II. The rate of acetylthiocholine hydrolyses by water extracts from pollen or pistils (in brackets) of various clones *Petunia hybrida*, differing in male fertility without and with the addition of the cholinesterase inhibitors. S.E. \pm 2%.

Clone	The rate of acetylthiocholine hydrolysis ($\mu\text{mol kg}^{-1}$ fresh mass $^{-1}$ s $^{-1}$)		
	Control	+ Physostigmine 10^{-5} M	+ Neostigmine 10^{-5} M
Self-compatible	1400 (492)	93 (307)	0 (330)
Self-incompatible	330 (50)	280 (22)	175 (0)
Sterile	25 (410)	300 (305)	230 (307)

90

Control of acetylcholinesterase gene expression in tissue-cultured skeletal muscle: Opposite regulation by protein kinases A and C

S.G. Rossi, S. Katz, R.L. Rotundo

Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, Florida 33136, USA

Introduction

The expression of acetylcholinesterase (AChE) in nerves and muscle is regulated by cellular activity, such as the frequency of membrane depolarization, and by signals from pre-synaptic cells. The specific signalling pathways involved, however, are still poorly defined. We have used primary cultures of quail muscle cells to study the regulation of AChE because they express both the globular and collagen-tailed AChE forms, and because they organize a subset of these on their surface in the form of clusters associated with other extracellular matrix components. Using quail-mouse mosaic myotubes, derived from the fusion of quail and mouse C2/C12 myoblasts, and avian-specific anti-AChE antibodies, we showed that AChE molecules expressed by a given quail nucleus are selectively targeted to and retained on the region of the fiber surface overlying that nucleus (Rossi and Rotundo, 1992). Once secreted, collagen-tailed AChE molecules interact transiently with heparan sulfate proteoglycans on the basal lamina prior to more permanent immobilization (Rossi and Rotundo, 1996). In vivo, specific binding sites exist at the adult neuromuscular junction for the localization of collagen-tailed AChE forms (Rotundo et al., 1997). Together, these studies indicated that synthesis, targeting, and localization of AChE is compartmentalized, and suggested that signals generated at the plasma membrane overlying individual nuclei may be responsible for locally regulating AChE expression. The questions then become: "what is the nature of the signals" and "at what level are they acting?"

Models for gene regulation at the neuromuscular junction have implicated protein kinase C (PKC), protein kinase A (PKA), and Ca^{2+} (reviewed in Duclert and Changeux, 1995), while stabilization of the acetylcholine receptors also may involve signaling through an adenylcyclase-linked pathway (Xu and Salpeter, 1995). In support of these hypotheses a specific isoform of PKC, PKC δ , has been localized immunocytochemically to the NMJ (Hilgenberg et al., 1996), and in situ hybridization and immunocytochemical analysis have revealed that a regulatory subunit of cAMP-dependent protein kinase (PKA) accumulates at the neuromuscular junction (Imaizumi-Scherrer et al., 1996). Regulation of the AChE gene and/or protein expression through signaling pathways that convey information about membrane activity have been previously investigated in several laboratories (De la Porte et al., 1984; Fernandez-Valle and Rotundo, 1989; Valette and Mas-soulié, 1991; Godinho et al., 1994; Griffman et al., 1997).

The regulation of AChE is compartmentalized

To determine whether the signaling pathways in multinucleated skeletal muscle fibers are compartmentalized, we designed tissue-culture chambers capable of isolating the medium overlying small regions of individual myotubes. The myotubes were grown on scratched coverslips so that the fibers formed in paralleled arrays on the coverslip, and the chambers were sealed over them with silicone grease to create separate chambers. Drugs that affected AChE expression were then added to one side of the cham-

ber while the other remained as an untreated control (Rossi et al., abstract American Society for Neuroscience, 1994; and manuscript in preparation). In parallel, sets of conventional cultures were used to monitor the effects of the drugs on AChE expression. We observed a reduction in the number of cell surface AChE clusters per nucleus following muscle paralysis using tetrodotoxin (TTX) in both conventional cultures and chamber cultures, where TTX was applied on one side of the chamber and normal medium on the other side. In contrast, when scorpion venom was applied to cells in either the conventional or chamber cultures, there was an increase in AChE clusters per nucleus as a consequence of chronic membrane depolarization. To determine whether AChE mRNA regulation was also compartmentalized, myotubes in chamber cultures were treated with TTX or scorpion venom on one side of the chamber and normal medium on the other side. The AChE mRNA distribution was analyzed by in situ hybridization using a digoxigenin-labeled 760 nt cRNA probe complementary to a common region of the AChE catalytic subunit. The ratio of nuclei showing accumulated AChE mRNA to the total nuclei in myotubes was determined for each treatment condition. We observed 64% more AChE mRNA positive nuclei in the presence of TTX, and 30% less positive nuclei under scorpion venom treated regions of the myotubes compared to the control side, indicating that local membrane depolarization could locally regulate AChE transcript levels in the same myotube. The morphology of the myotubes was similar along the entire fiber length regardless of treatment, yet the propagation of spontaneous contraction was inhibited only in those fiber regions exposed to the drugs. We concluded that accumulation of AChE transcripts and protein in specific regions of the muscle fiber is due in part to regulation of transcription as a result of localized signal transduction pathways.

Transcriptional control of AChE in muscle

AChE transcripts first appear at the time of myoblast fusion and then continue to increase for a short period of time. After reaching peak levels about 5 days after plating, AChE mRNA levels begin to decrease concomitantly with the increase in spontaneous muscle contraction. We analyzed whether the accumulation of AChE mRNA in quail muscle cultures during development and differentiation resulted from increased transcription or stabilization of the transcript. We observed that, in tissue cultured quail muscle, AChE mRNA is regulated at the transcriptional level during myoblast fusion and differentiation (Rossi and Rotundo, manuscript in preparation). The half life of AChE mRNA remained unchanged during myotube formation and maturation, using either the specific RNA polymerase II inhibitor DRB or actinomycin D to inhibit transcription. These studies also suggest major inter-species differences between mouse C2/C12 cells (Fuentes and Taylor, 1993) and avian cells with respect to AChE mRNA regulation. We observed that the rate of AChE mRNA transcription increased during differentiation of the muscle

fibers, but decreased after the onset of spontaneous contraction, with the second change being blocked by TTX.

Regulation of AChE by second messengers

Several reports have indicated the involvement of PKA and PKC in regulating proteins that accumulate at the neuromuscular junction, such as AChR (Fontaine et al., 1987; Chahine et al., 1993; O'Mally et al., 1997; reviewed in Duclert and Changeux, 1995) and NCAM (Rafuse and Landmesser, 1996), suggesting that nerve-induced changes in second messenger pathways can modulate muscle gene expression. In the present studies we investigated the role of PKA and PKC in modulating AChE expression using avian skeletal muscle cultures. Forskolin, a potent stimulator of adenylate cyclase that catalyzes the formation of cAMP, thereby activating cyclic-AMP-dependent protein kinases, decreased total AChE activity by 44% following overnight treatment. Forskolin reduced the expression of total AChE activity in a concentration-dependent manner, with a half-maximal inhibition near 30 μ M. Similar results were obtained using 8-bromo cAMP and dibutyryl-cAMP, as well as inhibitors of phosphodiesterases. No effects of forskolin on total protein synthesis were observed. To determine whether the effects of forskolin and cAMP analogues could act at the level of AChE biosynthesis, cells were pre-treated overnight with these drugs. The total cell-associated AChE was then irreversibly inhibited using diisopropylfluorophosphate (DFP), and cells were allowed to synthesize new AChE for 2 h in complete medium plus or minus drugs. Under these conditions, the rate of appearance of AChE activity in forskolin-treated cultures was 40% of controls, suggesting that forskolin affects the rate of AChE translation. Sucrose gradient analysis of AChE forms showed that, even after 24 h of recovery, all the forms are decreased in cells treated with forskolin. The collagen-tailed form in forskolin-treated cultures was only 15% of that in the control group. In addition, the rate of AChE secretion was decreased in forskolin-treated cultures. Interestingly, there was no effect on either total AChE activity or newly synthesized enzyme after only 3 h of treatment, suggesting that new synthesis is required. RNase protection assays were used to quantify the levels of AChE mRNA under the experimental different conditions and showed that AChE activity decreased in parallel with the decreasing levels of AChE mRNA. When phorbol-12,13-didecanoate (PDD), an activator of protein kinase C, was added to the cultures AChE activity was elevated.

In summary, activation of PKA inhibited the expression of AChE whereas activation of PKC increased enzyme activity, suggesting that the accumulation of AChE transcripts and enzyme at the neuromuscular junction involves localized regulation of AChE transcription. Moreover, these studies suggest a much more precise level of control than had been expected for an enzyme whose levels appear to be constitutively expressed at the synapse. This work was supported by a grant from the NIH to R.L.R.

References

- Chahine KG, Baracchini E, Goldman D (1993) Coupling muscle electrical activity to gene expression via a cAMP-dependent second messenger system. *J. Biol. Chem.* 268, 2893-2898
- De la Porte S, Vigny M, Massoulié J, Koenig J (1984) Action of veratridine on acetylcholinesterase in cultures of rat muscle cells. *Develop. Biol.* 106, 450-456
- Duclert A, Changeux JP (1995) Acetylcholine receptor gene expression at the developing neuromuscular junction. *Physiol. Rev.* 75, 339-368
- Fernandez-Valle C, Rotundo RL (1989) Regulation of acetylcholinesterase synthesis and assembly by muscle activity: Effects of tetrodotoxin. *J. Biol. Chem.* 264, 14043-14049
- Fontaine B, Klarsfeld A, Changeux JP (1987) Calcitonin gene-related peptide and muscle activity regulate acetylcholine receptor α -subunit mRNA levels by distinct intracellular pathways. *J. Cell Biol.* 105, 1337-1342
- Fuentes ME, Taylor P (1993) Control of acetylcholinesterase gene expression during myogenesis. *Neuron* 10, 679-687
- Godinho RO, Trivedi B, Rotundo RL (1994) Regulation of acetylcholinesterase (AChE) expression in skeletal muscle by second messengers and electrical stimulation. Abstract American Society for Neurosci
- Grifman M, Arbel A, Ginzberg D, Glick D, Elgavish S, Shaanan B, Soreq H (1997) In vitro phosphorylation of acetylcholinesterase at non-consensus protein kinase A sites enhances the rate of acetylcholine hydrolysis. *Mol. Brain Research* 51, 179-187
- Hilgenberg L, Yearwood S, Milstein S, Miles K (1997) Neural influence on protein kinase C isoform expression in skeletal muscle. *J. of Neurosci.* 16, 4994-5003
- Imaizumi-Scherrer T, Faust DM, Bénichou JC, Hellio R, Weiss MC (1996) Accumulation in fetal muscle and localization to the neuromuscular junction of cAMP-dependent protein kinase A regulatory and catalytic subunits R1 α and C α . *J. Cell Biol.* 134, 1241-1254
- O'Malley JP, Moore CP, Salpeter MM (1997) Stabilization of acetylcholine receptors by exogenous ATP and its reversal by cAMP and calcium. *J. Cell Biol.* 138, 159-165
- Rafuse VF, Landmesser LT (1996) Contractile activity regulates isoform expression and polyacylation of NCAM in cultured myotubes: involvement of Ca²⁺ and protein kinase C. *J. Cell Biol.* 132, 969-983
- Rossi SG, Rotundo RL (1992) Cell surface acetylcholinesterase molecules on multinucleated myotubes are clustered over the nucleus of origin. *J. Cell Biol.* 119, 1657-1667
- Rossi SG, Rotundo RL (1996) Transient interactions between collagen-tailed acetylcholinesterase and sulfated proteoglycans prior to immobilization on the extracellular matrix. *J. Biol. Chem.* 271, 1979-1987
- Rossi SG, Vazquez AE, and Rotundo RL (1994) Local control of acetylcholinesterase expression in skeletal muscle. Abstract American Society for Neurosci
- Rotundo RL, Rossi SG, Anglistter L (1997) Transplantation of quail collagen-tailed acetylcholinesterase onto the frog neuromuscular synapse. *J. Cell Biol.* 136, 367-374
- Vallette FM, Massoulié J (1991) Regulation of the expression of acetylcholinesterase by muscular activity in avian primary cultures. *J. of Neurochem.* 56, 1518-1525
- Xu R, Salpeter MM (1995) Protein kinase A regulates the degradation rate of Rs acetylcholine receptors. *J. of Cell. Physiol.* 165, 30-39

91

Effect of gestational nicotine exposure on hippocampal intrinsic cholinergic neurons

U. Sabherwal, K. Narayanan

Department of Anatomy, AIIMS, New Delhi, 110029, India

Learning and memory deficits and disorders of motor activity have been reported in children exposed to in utero nicotine as a result of maternal smoking during pregnancy¹. Neurobehavioral disturbances have been noticed in animal models of prenatal nicotine exposure². In an earlier morphological study on hippocampal neurons, distinct morphological changes in the pyramidal neurons of Ammon's horn and granule cells of dentate gyrus were observed in postnatal period after gestational nicotine insult. There was a significant reduction in neuronal size and dendritic arbor, by light microscopy; dilatation and membrane disruption of Golgi cisternae along with microvacuolation of rough endoplasmic reticulum by electron microscopy³. These neurotoxic effects on hippocampal neurons are important as the hippocampus plays a major role in spatial learning and memory.

In order to explore effects of prenatal nicotine on hippocampus further, intrinsic cholinergic neurons of hippocampus were studied in timed pregnant Wistar female rats (125–150 g) who were subjected to intraperitoneal nicotine (2.5 mg/kg/day in two divided doses), from gestation day 6 to term. Pups were delivered normally and their brains were dissected after perfusion fixation. Coronal sections of the dorsal hippocampal region were studied for intrinsic cholinergic neurons, from postnatal (PD) 40 to 80, by immunohistochemical techniques using monoclonal antibody against choline acetyl transferase (ChAT), (Boehringer Mannheim) and localising neurons by the ABC method. Cholinergic neurons were identified in Ammons' horn CA1 region (in strata oriens, pyramidal, radiatum and lacunosum moleculare) and in hilar region of dentate gyrus. Stereologic analysis of ChAT immunoreactive neurons was done on a Leica QC 500 Image Analysis System. Count of cholinergic neurons and their area was significantly reduced ($P < 0.001$) in nicotine-exposed animals at PD 80. Cholinergic neurons of hippocampus modulate the activity of pyramidal neurons of Ammons' horn, which in turn influence other cortical regions; hence these morphological alterations ob-

served in non-pyramidal ChAT immunopositive neurons are significant.

These morphological effects on hippocampal neurons correlate well with the Openfield motor activity of the animals recorded on Video path analyzer (Coulbourn Instruments). Total distance travelled, speed and duration of locomotion during a 30-min time period were significantly less in the experimental animals. Reduced hippocampal cholinergic activity as a result of gestational nicotine exposure during vulnerable period of neurogenesis is responsible for motor dysfunctions observed in the present study.

These results provide morphological evidence of disruption of cholinergic activity in late developing region of brain, hippocampus, produced by gestational nicotine insult. It has been reported that nicotine interacts with nicotinic cholinergic receptors in developing brain producing discoordination of target cell differentiation and causes premature shift from replication to differentiation as a result of nicotinic receptor stimulation on target cells directly or via release of catecholamines indirectly⁴. No such morphological study has been reported correlating intrinsic cholinergic neurons of hippocampus with the neurobehavioral dysfunctions.

References

1. Butler NR, Goldstein H (1973) Smoking in pregnancy and subsequent child development. *Br Med J* 4, 573–574.
2. Yanai J, Pick CG, Rogel-Fuchs Y, Zahalaka EA (1991) Alterations in the septohippocampal cholinergic receptors and related behaviour after prenatal exposure to nicotine. In Adlkofer F, Thureau K eds. *Effects of nicotine on biological system*. Basel: Birkhauser Verlag, 465–469.
3. Roy TS and Sabherwal U (1998) Effects of gestational nicotine exposure on hippocampal morphology. *Neurotoxicology and Teratology* 20, 1–9.
4. Navarro HA, Seidler FJ, Eylers JP, Baker FE, Dobbins SS, Lappi SE, Slotkin TA (1989) *J Pharmacol Exp Ther* 251, 894–900.

92

Analysis of developmental evolution of synapses in CGRP knock-out mice

A.-M. Salmon^a, S. Sekine^b, M. Piccioletto^c, I. Damaj^d, J.-P. Changeux^a

^aCNRS-UA D1284 "Neurobiologie Moléculaire", Institut Pasteur, Paris, France

^bDept. of Molecular Genetics, Tokyo Research Laboratories, Japan

^cLaboratory of Molecular Neurobiology and Pharmacology, Yale University, New Haven; ^dVirginia Commonwealth University, Richmond, USA

Calcitonin gene-related peptide (CGRP) is a neuropeptide widely localized in central and peripheral nervous systems. Its different ascribed actions include modulations of the enteric autonomous system, cardiovascular effects, vasodilatation and inflammation phenomena, and stimulation of acetylcholine release particularly from motor nerve endings at neuromuscular junctions. Also CGRP is potentially involved in pain sensitivity to different stimuli. Taken together, these observations define the role of CGRP as a trophic or modulatory factor in neuron or neuronal-muscle cell

interactions. To further precise the role of CGRP we made knock-out mice (CGRP^{-/-}) by exon 5 specific mutation of the calcitonin/CGRP gene.

Homozygous CGRP^{-/-} mice live to adult-hood, reproduce normally and show no abnormalities when observed in their cage. However, preliminary behavioural analysis indicates some differences in base line nociception and morphine sensitivity between wild type and knock-out mice latencies when using heat tests.

93

Transgenic mammary gland expression of 'readthrough' human AChE: A model system for cholinesterase regulation in mammalian body fluids

A.Y. Salmon^a, M. Sternfeld^a, D. Ginsberg^a, J. Patrick^b, H. Soreq^a

^aThe Life Sciences Inst., The Hebrew University, Jerusalem 91904, Israel

^bBaylor College of Medicine, Division of Neuroscience, One Baylor Plaza, Houston, TX 77030-3498, USA

Apart from their well known association with cholinergic synapses, the acetylcholine hydrolyzing enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) appear in body fluids (e.g., plasma, amniotic and cerebrospinal fluids) where their function(s) are not fully understood. To investigate the molecular mechanisms underlying AChE and BuChE secretion into body fluids, we studied mammary gland expression of cholinesterases in two lines of transgenic mice overexpressing the human (h) 3'-alternatively spliced 'readthrough' AChE mRNA transcript including the 14 pseudointron and the 3' terminal exon E5, (hAChE-I4 /E5) directed by the cytomegalovirus (CMV) efficient promoter. Two additional lines expressed catalytically active (Beeri et al., 1995, 1997) or insert-inactivated AChE terminated with the brain characteristic C-terminus translated from exon 6 (hAChE-E6 and hInAChE-E6; Sternfeld et al., 1998). Non-transgenic FVB/N mice served as controls. In mammary gland alveoli of hAChE-I4/E5 transgenic mice, *in situ* hybridization demonstrated pronounced labeling of hAChE-I4/E5 mRNA transcripts, considerably higher than the endogenous mouse (m) AChE-I4/E5 mRNA transcripts detected in both wild type FVB/N mice and the h-AChE-E6 transgenic mice. The capacity of milk cholinesterases to hydrolyse acetylthiocholine (ATCh) was tested in the absence or presence of the BuChE-specific inhibitor iso OMPA throughout the first 2 weeks after delivery. In both control mice and transgenics expressing active h-AChE-E6, BuChE was the predominant cholinesterase and hydrolysed ca. 35 nmol ATCh/min/ μ L milk. Iso OMPA (10^{-5} M) inhibited over 80% of this activity. Transgenics expressing h-InAChE-E6 displayed considerably lower levels of BuChE in milk, with activities increasing from virtually undetected amounts to 7.5 and 15 nmol/min/ μ L at days 7, 11 and 14

post-partum. This suggested competitive inhibition of host AChE production in a manner dependent on lactation time. Transgenic mice expressing 25-fold higher levels of h-AChE-I4/E5 in muscle displayed similarly higher AChE activities also in milk. Another line, with 350-fold transgenic h-AChE-I4/E5 levels in muscle, revealed increases of up to 160-fold higher than control amounts in milk, with two-fold post-delivery elevations from day 7 to 14. Non-denaturing gel electrophoresis followed by cytochemical staining demonstrated a complex migration pattern with a pronounced band co-migrating with h-AChE-I4/E5 produced in *Xenopus* oocytes. Altogether, the transgenic enzyme thus reached higher concentrations than the 50 nM cholinesterases present in normal human blood. Our findings present novel transgenic models for studying the secretion, control of production and biochemical properties of cholinesterases in mammalian milk.

References

- Beeri, R., Andres, C., Lev-Lehman, E., Timberg, R., Huberman, T., Shani, M. and Soreq, H. (1995) Transgenic expression of human acetylcholinesterase induces progressive cognitive deterioration in mice. *Curr. Biol.*, 5, 1063-1071.
- Beeri, R., LeNovere, N., Mervis, R., Huberman, T., Grauer, E., Changeux, J.P. and Soreq, H. (1997). Enhanced hemicholinium binding and attenuated dendrite branching in cognitively impaired AChE-transgenic mice. *J. Neurochem.* 69, 2441-2451.
- Sternfeld, M., Ming, G-L., Song, H-J., Sela, K., Poo, M-M. and Soreq, H. (1998). Acetylcholinesterase enhances neurite growth and synapse development through alternate contributions of its hydrolytic capacity, core protein and variable C-termini. *J. Neurosci.*, 18, 1240-1249.

94

Implication of a multisubunit Ets related transcription factor in synaptic expression of the nicotinic acetylcholine receptor

L. Schaeffer, A. de Kerchove d'Exaerde, N. Duclert, M. Huchet-Dymanus, J.-P. Changeux

CNRS-UA D1284 'Neurobiologie Moléculaire', Institut Pasteur, Paris, France

In adult muscle, transcription of the nicotinic acetylcholine receptor (AChR) is restricted to the nuclei located at the neuromuscular junction. The N-box, a new promoter element, was recently identified and was shown to contribute to this compartmentalized synaptic expression in the case of the AChR delta and epsilon subunits. Here, we first demonstrate that the N-box mediates transcriptional activation in cultured myotubes and identifies the transcription factor that binds to the N-box in myotubes and in adult muscle as an heterooligomer. The alpha subunit of GABP (GA binding protein) belongs to the Ets family of transcription factors, whereas the beta-subunit shares homology with IxB and *Drosophila* Notch protein. GABP binding specificity to

mutated N-box *in vitro* strictly parallels the sequence requirement for beta galactosidase targeting to the endplate *in vivo*. *In situ* hybridization studies reveal that both GABP subunits mRNAs are abundant in mouse diaphragm, with a preferential expression of the alpha subunit at motor endplates. In addition, heregulin increases GABP alpha protein levels and regulates the phosphorylation of both GABP subunits in cultured chick myotubes. Finally, dominant negative mutants of either GABP alpha or GABP beta block heregulin elicited transcriptional activation of the AChR delta and epsilon genes. These findings establish the expected connection with a synaptic trophic factor which contributes to the accumulation of AChR subunits mRNAs at the motor endplate.

95

Mechanisms which regulate the cholinergic phenotype in sympathetic, central cholinergic and spinal motoneurons

M. Sendtner

Klinische Forschergruppe Neuroregeneration, Dept. of Neurology, University of Würzburg, Josef-Schneider-Str. 11, 97080 Würzburg, Germany

Besides their function in supporting neuronal survival, neurotrophic factors influence various functional properties of responsive neurons. In cell culture, neurotrophic factors of the CNTF/LIF family can switch the transmitter phenotype of sympathetic neurons from adrenergic to cholinergic (Schotzinger and Landis, 1990; Saadat et al., 1989; Ernsberger et al., 1997), and choline acetyl transferase expression both in central cholinergic neurons as well as in spinal motoneurons is influenced by neurotrophic factors (Gnahn et al., 1983; Magal et al., 1991; Williams et al., 1996; Wong et al., 1993; Alderson et al., 1996). Although these mechanisms have been studied in detail in cell culture, it is not clear whether the same factors also regulate cholinergic transmitter production of these neurons in vivo. Ciliary neurotrophic factor and leukemia-inhibitory factor are not expressed within the innervated target region of cholinergic neurons such as sweat gland and skeletal muscle (Stöckli et al., 1991; Banner and Patterson, 1994). Instead, they are found in Schwann cells within the peripheral nerve and astrocytes within the central nervous system. In particular after peripheral nerve or mechanical brain lesion, the production of LIF is increased, and CNTF can be released in order to act on lesioned cholinergic neurons (Sendtner et al., 1997). Currently, mouse mutants lacking the genes for neurotrophic factors (DeChiara et al., 1995; Li et al., 1995; Sendtner et al., 1996; Francis et al., 1997) are analyzed to define the physiological endogenous role of these factors in regulating the cholinergic phenotype of these neurons.

References

- Alderson RF, Wiegand SJ, Anderson KD, Cai N, Cho JY, Lindsay RM, Altar CA (1996) Neurotrophin-4/5 maintains the cholinergic phenotype of axotomized septal neurons. *Eur J Neurosci* 8, 282–290
- Banner LR, Patterson PH (1994) Major changes in the expression of the mRNAs for cholinergic differentiation factor/Leukemia inhibitory factor and its receptor after injury to adult peripheral nerves and ganglia. *Proc Natl Acad Sci USA* 91, 7109–7113
- DeChiara TM, Vejsada R, Poueymirou WT, Acheson A, Suri C, Conover JC, Friedman B, McClain J, Pan L, Stahl N, et al (1995) Mice lacking the CNTF receptor, unlike mice lacking CNTF, exhibit profound motor neuron deficits at birth. *Cell* 83, 313–322
- Ernsberger U, Patzke H, Rohrer H (1997) The developmental expression of choline acetyltransferase (ChAT) and the neuropeptide VIP in chick sympathetic neurons: evidence for different regulatory events in cholinergic differentiation. *Mech Dev* 68, 115–126
- Francis NJ, Asmus SE, Landis SC (1997) CNTF and LIF are not required for the target-directed acquisition of cholinergic and peptidergic properties by sympathetic neurons in vivo. *Dev Biol* 182, 76–87
- Gnahn H, Hefti F, Heumann R, Schwab ME, Thoenen H (1983) NGF-mediated increase of choline acetyltransferase (ChAT) in the neonatal rat forebrain: evidence for a physiological role of NGF in the brain. *Dev Brain Res* 9, 45–52
- Li M, Sendtner M, Smith A (1995) Essential function of LIF receptor in motor neurons. *Nature* 378, 724–727
- Magal E, Burnham P, Varon S (1991) Effects of ciliary neuronotrophic factor on rat spinal cord neurons in vitro: survival and expression of choline acetyltransferase and low-affinity nerve growth factor receptors. *Dev Brain Res* 63, 141–150
- Saadat S, Sendtner M, Rohrer H (1989) Ciliary neurotrophic factor induces cholinergic differentiation of rat sympathetic neurons in culture. *J Cell Biol* 108, 1807–1816
- Schotzinger RJ, Landis SC (1990) Acquisition of Cholinergic and Peptidergic Properties by Sympathetic Innervation of Rat Sweat Glands Requires Interaction with Normal Target. *Neuron* 5, 91–100
- Sendtner M, Götz R, Holtmann B, Escary J-L, Masu Y, Carroll P, Wolf E, Brehm G, Brulet P, Thoenen H (1996) Cryptic physiological trophic support of motoneurons by LIF disclosed by double gene targeting of CNTF and LIF. *Current Biol* 6, 686–694
- Sendtner M, Götz R, Holtmann B, Thoenen H (1997) Endogenous ciliary neurotrophic factor is a lesion factor for axotomized motoneurons in adult mice. *J Neurosci* 17, 6999–7006
- Stöckli KA, Lillien LE, Näher-Noe M, Breitfeld G, Hughes RA, Thoenen H, Sendtner M (1991) Regional distribution, developmental changes and cellular localization of CNTF-mRNA and protein in the rat brain. *J Cell Biol* 115, 447–459
- Williams LR, Inouye G, Cummins V, Pelleymounter MA (1996) Glial cell line-derived neurotrophic factor sustains axotomized basal forebrain cholinergic neurons in vivo: dose-response comparison to nerve growth factor and brain-derived neurotrophic factor. *J Pharmacol Exp Ther* 277, 1140–1151
- Wong V, Arriga R, Ip N, Lindsay RM (1993) The neurotrophins BDNF, NT-3 and NT-4/5, but not NGF, upregulate the cholinergic phenotype of developing motoneurons. *Europ J Neurosci* 5, 466–474

96

An engineered short-chain toxin with higher affinity for the neuronal $\alpha 7$ receptor

D. Servent^a, S. Antil^a, G. Mourier^a, P.J. Corringer^b, A. Ménez^a

^aCEASaclay, Département d'Ingénierie et d'Etudes des Protéines, 91191 Gif-sur-Yvette;

^bLaboratoire de Neurobiologie Moléculaire, Institut Pasteur, 75724 Paris cedex 15, France

Elapid and hydrophid snakes synthesize curare-mimetic toxins which block nicotinic acetylcholine receptor from *Torpedo* with high affinities. These toxins are usually classified as short-chain (60–62 residues and 4 disulfide bonds) and long-chain (66–74

residues and 5 disulfide bonds) (Endo and Tamiya, 1991). On the basis of electrophysiological and binding experiments, we recently demonstrated that only long-chain toxins possessing 5 disulfide bonds are capable of blocking the $\alpha 7$ neuronal acetylcholine re-

Table I. Affinity constants of wild-type and modified curare-mimetic toxins on the chimeric $\alpha 7$ -5HT3 receptor expressed in HEK cells.

	<i>K_d on the $\alpha 7$ receptor</i>
	1 nM
α -Bgtx	5 nM
α -Cbtx	2 μ M
Toxin- α	35 nM
Bgtx (C29S-C33S)	120 nM
Cbtx (C26S-C30S)	100 nM

ceptor with high affinities ranging between 1 to 10 nM (Servent et al., 1997). The short-chain toxins recognize this receptor with much lower affinities, their K_d values being at least three orders of magnitude higher than those of long-chain toxins. Selective reduction of the extra disulfide bond of the long-chain toxin, followed by a specific modification of the free cysteines with a bulky chemical reagent, further confirmed the critical role of this particular region for curare-mimetic toxins to bind to the neuronal $\alpha 7$ receptor with high affinity.

We now tentatively investigated the binding contribution of this toxin region to the neuronal $\alpha 7$ receptor by synthesizing a

number of toxin variants. At first, we synthesized genetically or chemically two derivatives of the long-chain α -cobratoxin and α -bungarotoxin, in which the extra two half-cystines were replaced by serine residues. The affinities of these derivatized toxins for the $\alpha 7$ receptor decreased by 25-fold and 35-fold respectively with K_d equal to 120 and 35 nM (table I), whereas their affinity constants for the Torpedo receptor remained virtually unchanged. Then, we selected a short-chain toxin having low affinity ($K_d = 2 \mu$ M) for the $\alpha 7$ receptor and by a chemical approach we introduced in it an extra disulfide and its associated loop, at a location homologous to that observed in long-chain toxins. The resulting chimeric toxin displayed a K_d equal to 100 nM (table I), which corresponds to an affinity increase of 20-fold. Therefore, we have not only unambiguously localized a region that is associated with the capacity of toxins to recognize the neuronal $\alpha 7$ receptor but we also determined its contribution to this recognition.

References

- Endo T, Tamiya N (1991) in Snake Toxins (Harvey, AL ed) Pergamon Press, New York 259–302
 Servent D, Winckler-Dietrich V, Hu HY, Kessler P, Drevet P, Bertrand P, Ménez A (1997) Only snake curare-mimetic toxins with a fifth disulfide bond have high affinity for the neuronal $\alpha 7$ nicotinic receptor. *J. Biol. Chem.* 272, 24279–24286

97

Nicotinic and muscarinic modulation of epileptic activities in the rat GABA withdrawal syndrome

C. Silva-Barrat, M. Szente, C. Menini, J. Velluti, J. Champagnat

Laboratoire d'Epilepsie Experimentale, UMR 9923, CNRS, Faculte Pitie-Salpetriere, 91, bvd de l'Hopital, 75634 Paris cedex 13, France

The γ -aminobutyric acid (GABA) withdrawal syndrome (GWS) is a focal epilepsy consecutive to the interruption of a chronic GABA infusion into the somatomotor cortex (Brailowsky et al., 1988). In neocortical slices obtained from rats presenting GWS, epileptic-like bursting activities are induced in pyramidal cells by intracellular depolarizing current injection (intrinsic bursting) and/or by white matter (WM) stimulation (synaptic bursting; Silva-Barrat et al., 1989, 1992). The aim of the present study is to analyse the role of cholinergic neurotransmission in this model of epilepsy. We tested the effects of acetylcholine and carbacol (ACh and Cch, acting on both muscarinic and nicotinic receptors) on intrinsic and synaptic bursting of pyramidal cells in slices obtained from GWS (epileptic) rats. These effects were compared to those observed on regular spiking cells from control (non-epileptic) rats. The respective contribution of muscarinic and nicotinic

receptors was assessed using selective agonists methyl-acetylcholine (mACh, muscarinic), nicotine, and the muscarinic antagonist atropine. When applied alone, atropine did not significantly modify the membrane potential of the studied neurons.

All agonists in both epileptic and non-epileptic preparations caused a slow depolarization of the membrane potential associated with an increase of action potential (AP) firing induced by depolarizing current injection and, in most cells, with an increase of the input resistance. This effect which was stronger with nicotine inducing spontaneous AP firing, was considered unrelated to the epileptic behaviour. The excitatory effect of nicotine was associated with an increase of the afterdepolarization following synaptically induced APs. This generated additional APs even if the membrane potential was repolarized by current injection to control levels. In contrast, mACh decreased the afterdepolarization following APs.

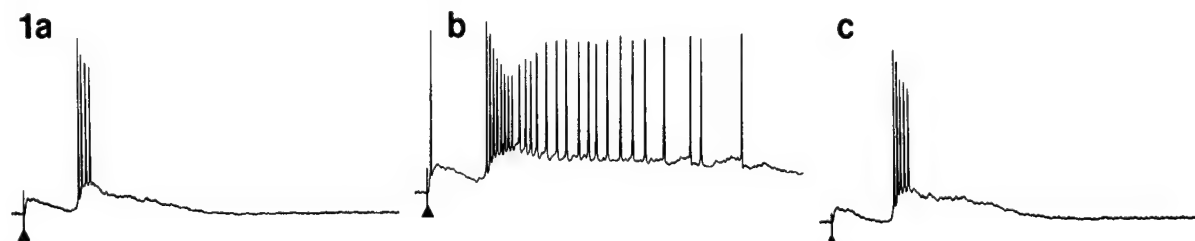


Figure 1. Potentiation by nicotine (b) of synaptically induced burst in an epileptic rat (control: a). The synaptic bursts persisted even when the membrane potential was brought back to resting level (c).

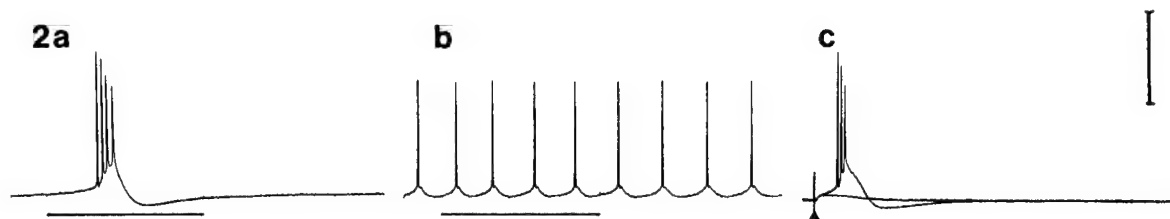


Figure 2. Transformation by mACh of intrinsic bursts induced by depolarizing current injection (a) into a tonic AP firing (b). c. Superimposed synaptically induced responses in control condition (burst) and during mACh application (depression of burst). Horizontal bar: pulse of depolarizing current (250 ms, 0.02 nA). Vertical bar: amplitude (50 mV). Triangles: synaptic stimulation.

In epileptic preparations, synaptic bursting was diminished or blocked by Ach and CCh. This effect was ascribed to muscarinic mechanisms because it was obtained with mACh (figure 2c) and reduced by atropine. When CCh was applied in combination with atropine, we observed a small hyperpolarization of the membrane potential associated with a diminished response to depolarizing current injection and to synaptic stimulation. Contrarily to the other cholinergic agonists, nicotine potentiated synaptic bursts (figure 1a, b): under nicotine, the synaptic bursts persisted when the membrane potential was either depolarized by nicotine or brought back at resting level by current injection (figure 1c).

In bursting pyramidal cells, Ach and CCh increased the number of bursts induced by depolarizing current injection. This effect was also induced by nicotine that favoured the appearance of spontaneous bursting activity. In contrast, mACh, when applied on bursting cells of GWS rats, provoked a transformation of bursts into a tonic AP firing associated with the slow depolarization (figure 2a,b). When mACh was applied in combination with atropine, the membrane potential was not significantly modified and the epileptic bursts were activated instead of being transformed in a tonic discharge of APs.

These results suggest that both cholinergic receptors are involved in the epileptogenic GWS with opposite effects. The activation of metabotropic muscarinic receptors (by mACh) depressed the 'epileptic like' discharges while the activation of ionotropic nicotinic receptors (by nicotine) facilitated them. The simultaneous activation of both types of receptors (by Ach or CCh) had a complex effect favouring intrinsic bursting and depressing synaptic bursts. Interestingly, CCh was more efficient than Ach

and, in some cells, provoked oscillatory variations of the membrane potential associated with an important activation of AP firing. This observation might therefore provide a basis for a cholinergic modulation of the epileptic activity through the simultaneous activation of muscarinic and nicotinic receptors. Our results are also supported by recent findings showing that, in the GABA-injected cortical area, the number of choline acetyltransferase immunopositive neurones is significantly more important in GWS rats than in control rats (Araneda et al., 1994). Given that the cholinergic innervation of the cerebral cortex in normal rats mainly comes from the basal forebrain, the preceding observation suggests the appearance in epileptic rats of neurones neosynthesizing choline acetyltransferase. This work was supported by a grant from ECOS, U95E01.

References

- Araneda S; Silva-Barrat C; Menini C; Naquet R. High expression of noradrenaline, choline acetyltransferase and glial fibrillary acidic protein in the epileptic focus consecutive to GABA withdrawal. An immunocytochemical study. *Brain Res.* 1994, 655:135-146.
- Brailowsky S; Kunitomo M; Menini Ch; Silva-Barrat C; Riche D; Naquet R. The GABA-withdrawal syndrome: a new model of focal epileptogenesis. *Brain Res.* 1988,442:175-179.
- Silva-Barrat C., Araneda S., Menini Ch., Champagnat J., and Naquet R. Burst generation in neocortical neurons after GABA withdrawal in the rat. *J. Neurophysiol.* 1992,67:715-727.
- Silva-Barrat C., Champagnat J., Brailowsky S., Menini Ch. and Naquet R. Relationship between tolerance to GABA agonist and bursting properties in neocortical neurons during GABA-withdrawal Syndrome. *Brain Res.* 1989, 498:289-298.

Inhibition of *Electrophorus* acetylcholinesterase by monoclonal antibodies

S. Simon^a, A. Le Goff^a, Y. Frobert^b, J. Grassi^b, J. Massoulié^a

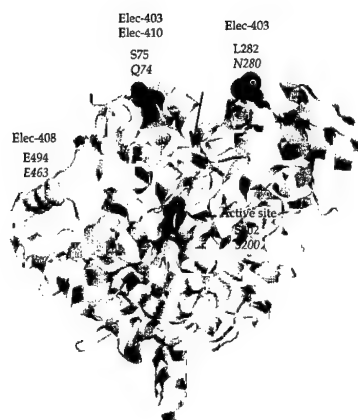
^aLaboratoire de Neurobiologie Moléculaire et Cellulaire, École Normale Supérieure, 46 rue d'Ulm, Paris, France

^bCEA, service de Pharmacologie et d'Immunologie, Centre d'Etudes de Saclay, Gif-sur-Yvette, France

In acetylcholinesterase (AChE), acetylcholine is guided along aromatic residues to the active site, located at the bottom of the catalytic gorge addin (Sussman et al., 1991). The way of exit of the products is unknown and this led to the hypothesis of a 'back door', opening at the bottom of the gorge (Gilson et al., 1994). However, this was not demonstrated experimentally and none of the known inhibitors binds to this region. AChE activity is irreversibly blocked by active site inhibitors or by ligands that bind

to the peripheral site, located at the surface of the protein near the entrance of the gorge.

We studied *Electrophorus electricus* AChE for several reasons. Firstly, it has been used extensively for studies of acetylcholine hydrolysis. Secondly, its catalytic turn-over is the highest among studied AChEs. Thirdly, three monoclonal antibodies that inhibit specifically its catalytic activity have been isolated (Rémy et al., 1995). Two of them recognized overlapping but different epitopes



(Elec-403 and Elec-410); their binding is competitive with that of peripheral site ligands. The third antibody (Elec-408) binds to another site and its inhibitory effect is additive with the other two antibodies, thus defining a new regulatory site on the enzyme. In order to determine the binding sites of these antibodies, we constructed chimeric molecules composed of parts of *Electrophorus* AChE and rat AChE (unrecognized by the antibodies). The sensitivity of these chimeras to the antibodies allows us to define

regions implicated in their recognition. By site directed mutagenesis, we have been able to abolish the inhibitory effects of each of the three antibodies. These experiments confirm that two antibodies bind to the peripheral site and recognize distinct epitopes with at least one common residue. The third antibody binds to another part of the enzyme, corresponding to the theoretical 'back door'. We hope that an analysis of the inhibition mechanism of this antibody will allow us to confirm the 'back door' hypothesis and to analyze its possible function during the catalytic cycle.

A ribbon three-dimensional model of *Electrophorus* AChE. The entrance of the catalytic gorge is indicated by an arrow; the active serine of the catalytic site and residues involved in the binding of the three antibodies are shown in space filling view.

References

- Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L. and Silman, I. (1991) Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein. *Science* 253, 872-879.
- Gilson, M. K., Straatsma, T. P., Mc Cammon, J. A., Ripoll, D. R., Faerman, C. H., Axelsen, P. H., Silman, I. and Sussman, J. L. (1994) Open "back door" in a molecular dynamics simulation of acetylcholinesterase. *Science* 263, 1276-1278.
- Rémy, M.H., Frobert, Y. and Grassi, J. (1995) Characterization of monoclonal antibodies that strongly inhibit *Electrophorus electricus* acetylcholinesterase. *J. Biochem.* 231, 651-658.

99

Integrin activation is required for protein tyrosine phosphorylation induced by activation of muscarinic m3 receptors

B.E. Slack

Department of Pathology and Laboratory Medicine, Boston University School of Medicine,
85 East Newton Street, Rm M1007, Boston, MA 02118, USA

Muscarinic receptors are characterized by seven transmembrane domains, and signal by coupling to heterotrimeric GTP binding (G) proteins. Of the five muscarinic receptor isoforms that have been cloned (Peralta et al., 1987; Bonner et al., 1988); three (m1, m3, and m5) stimulate phosphatidylinositol 4,5 bisphosphate (PIP2) breakdown, and two (m2 and m4) inhibit adenyl cyclase (Peralta et al., 1988; Sandmann et al., 1991). Activation of PIP2-coupled receptors results in hydrolysis of PIP2 to form diacylglycerol and inositol-1,4,5-trisphosphate, with subsequent activation of protein kinase C (PKC). Muscarinic receptors activate other kinases as well, including mitogen-activated protein kinase (Crespo et al., 1994), and tyrosine kinases of the Src family (Wan et al., 1996; Tsai et al., 1997). A variety of proteins are tyrosine phosphorylated following muscarinic receptor activation, including the focal adhesion-associated proteins paxillin and focal adhesion kinase (FAK) (Gutkind and Robbins, 1992; Petryniak et al., 1996).

Focal adhesions are attachment sites found in cultured cells; at these sites the extracellular domains of cell-surface integrins bind to immobilized extracellular matrix (ECM) proteins such as fibronectin (Burrige et al., 1988). Focal adhesion formation is initiated experimentally by the plating of cell suspensions onto immobilized ECM proteins (Burrige et al., 1997), and results in clustering of the integrins, and the association of their intracellular domains with cytoskeletal proteins that anchor polymerized actin filaments (stress fibers) to these sites. A number of signaling proteins are recruited to focal adhesions, including the adapter protein paxil-

lin, and the tyrosine kinase FAK (Clark and Brugge, 1995). Thus focal adhesions have both structural and signaling functions. Focal adhesions are also formed following the addition of growth factors or G protein-coupled receptor ligands to quiescent cells (Ridley and Hall, 1992; Seufferlein and Rozengurt, 1994). Exposure either to immobilized ECM proteins, or to soluble receptor ligands, results in transient tyrosine phosphorylation of similar sets of proteins, including tensin, p130^{cas}, paxillin and FAK (Burrige et al., 1997). In fibroblasts, G protein-coupled receptor-mediated tyrosine phosphorylation and focal adhesion formation are dependent on cytoskeletal integrity, on activation of the small molecular G-protein Rho, and on actomyosin contractility (Rankin et al., 1994; Chrzanowska-Wodnicka and Burrige, 1996; Sinnett-Smith et al., 1993).

The experiments described in this report were carried out in human embryonic kidney cells stably expressing muscarinic m3 receptors (HEK-m3). Treatment of serum-deprived monolayers of HEK-m3 cells with the muscarinic receptor ligand carbachol resulted in time- and concentration-dependent increases in tyrosine phosphorylation of the proteins paxillin and FAK (assessed by Western blot). The response approached maximal values within 10 min, and at a carbachol concentration of 1 μ M. Although tyrosine phosphorylation of paxillin and FAK elicited by a variety of receptor ligands in Swiss 3T3 cells was reported to be independent of PKC (Sinnett-Smith et al., 1993), the response to carbachol of HEK-m3 cells appeared to be partially dependent on this enzyme, since it was reduced in the presence of 2.5 μ M of

the specific PKC inhibitor GF109203X. Tyrosine phosphorylation of paxillin and FAK was also induced, although to a lesser extent, by phorbol 12-myristate 13-acetate, a direct activator of PKC; this response was abolished by the inhibitor.

An identical pattern of tyrosine phosphorylation was induced in serum-deprived HEK-m3 cells that were first placed in suspension, then allowed to attach to culture dishes coated with fibronectin. This response was not observed in cells adhering to dishes coated with poly-D-lysine. In agreement with results from other laboratories, tyrosine phosphorylation was inhibited when cells were allowed to adhere to fibronectin in the presence of soluble peptides containing the amino acid sequence arginine-glycine-aspartate (RGD), which mimics the integrin binding site on ECM proteins such as fibronectin. Interestingly, RGD peptides also reduced carbachol-mediated tyrosine phosphorylation in quiescent monolayers of HEK-m3 cells, suggesting that integrins might be direct downstream mediators of activated muscarinic receptors. Control peptides containing an inactive sequence (RGE) had no effect. The PKC inhibitor GF109203X did not alter tyrosine phosphorylation induced by adhesion to fibronectin, although it reduced the response to carbachol, suggesting that PKC lies upstream of integrins in the signaling pathway initiated by muscarinic receptor stimulation.

If integrin activation is necessary for muscarinic receptor-induced tyrosine phosphorylation, then the response should also require the presence of ECM proteins, which are necessary for integrin clustering and formation of focal adhesions. It is likely that ECM proteins were present in the monolayers used in the preceding experiments, since the cells were grown for several days in culture. Many cell types, including a number of kidney cell lines, secrete fibronectin and assemble it into a matrix; moreover, the $\alpha 1\beta 5$ integrin, which mediates cell adhesion to fibronectin, is expressed by HEK cells (Bodary and McLean, 1990). To test the dependence on ECM proteins of the response to carbachol, cells in suspension were plated on dishes coated with fibronectin or poly-D-lysine, then left undisturbed for 2.5 h, i.e., long-enough for the initial response to fibronectin to subside, but before substantial amounts of fibronectin could be secreted. The cells were then incubated in fresh medium in the absence or presence of carbachol for 20 min, and tyrosine phosphorylation was determined. As predicted by the earlier results, the response to carbachol was greatly attenuated in cells plated onto poly-D-lysine relative to those adherent to fibronectin.

As a final test of the hypothesis that integrins mediate muscarinic receptor signaling, the ability of muscarinic receptors to induce focal adhesion formation (an indicator of integrin activation) was examined using an immunofluorescence approach. Exposure to carbachol for 10 min induced the formation of stress fibers (visualized with fluorescent-conjugated phalloidin) and the appearance of focal adhesions (detected using an antibody to vinculin, a major component of these structures) in HEK-m3 cells.

In summary, the results suggest that stimulation of muscarinic m3 receptors elicits ECM-dependent clustering of integrins, with consequent formation of focal adhesions, and the recruitment and tyrosine phosphorylation of paxillin and FAK. PKC appears to mediate, at least in part, the activation of integrins by muscarinic receptor stimulation. Integrins thus represent novel intermediates in

muscarinic receptor-coupled signaling pathways. Supported by NIH grant NS30791. HEK-m3 cells were a generous gift from E. Peralta.

References

- Bodary SC and McLean JW (1990) The integrin $\beta 1$ subunit associates with the vitronectin receptor αv subunit to form a novel vitronectin receptor in a human embryonic kidney cell line. *J Biol Chem* 265:5938-5941.
- Bonner TI, Young AC, Brann MR and Buckley NJ (1988) Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron* 1:403-410.
- Burridge K, Fath K, Kelly T, Nuckolls G and Turner C (1988) Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Ann Rev Cell Biol* 4:487-525.
- Burridge K, Chrzanowska-Wodnicka M and Zhong C (1997) Focal adhesion assembly. *Trends in Cell Biol* 7:342-347.
- Chrzanowska-Wodnicka M and Burridge K (1996) Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol* 133:1403-1415.
- Clark EA and Brugge JS (1995) Integrins and signal transduction pathways: The road taken. *Science* 268:233-239.
- Crespo P, Xu N, Simonds WF and Gutkind JS (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein β gamma subunits. *Nature* 369:418-420.
- Gutkind JS and Robbins KC (1992) Activation of transforming G protein-coupled receptors induces rapid tyrosine phosphorylation of cellular proteins, including p125^{FAK} and the p130 v-src substrate. *Biochem Biophys Res Commun* 188:155-161.
- Peralta EG, Ashkenazi A, Winslow JW, Ramachandran J and Capon DJ (1987) Distinct primary structures, ligand-binding properties and tissue specific expression of four human muscarinic acetylcholine receptors. *EMBO J* 6:3923-3929.
- Peralta EG, Ashkenazi A, Winslow JW, Ramachandran J and Capon DJ (1988) Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. *Nature* 334:434-437.
- Petryniak MA, Wurtman RJ and Slack BE (1996) Elevated intracellular calcium concentration increases secretory processing of the amyloid precursor protein by a tyrosine phosphorylation-dependent mechanism. *Biochem J* 320:957-963.
- Rankin S, Morii N, Narumiya S and Rozengurt E (1994) Botulinum C3 exoenzyme blocks the tyrosine phosphorylation of p125FAK and paxillin induced by bombesin and endothelin. *FEBS Lett* 354:315-319.
- Ridley AJ and Hall A (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70:389-399.
- Sandmann J, Peralta EG and Wurtman RJ (1991) Coupling of transfected muscarinic acetylcholine receptor subtypes to phospholipase D. *J Biol Chem* 266:6031-6034.
- Seufferlein T and Rozengurt E (1994) Lysophosphatidic acid stimulates tyrosine phosphorylation of focal adhesion kinase, paxillin, and p130. Signaling pathways and cross-talk with platelet-derived growth factor. *J Biol Chem* 269:9345-9351.
- Sinnett-Smith J, Zachary I, Valverde AM and Rozengurt E (1993) Bombesin stimulation of p125 focal adhesion kinase tyrosine phosphorylation. Role of protein kinase C, Ca^{2+} mobilization, and the actin cytoskeleton. *J Biol Chem* 268:14261-14268.
- Tsai W, Morielli AD and Peralta EG (1997) The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity. *EMBO J* 16:4597-4605.
- Wan Y, Kurosaki T and Huang XY (1996) Tyrosine kinases in activation of the MAP kinase cascade by G-protein-coupled receptors. *Nature* 380:541-544.

100

Effect of pyridostigmine administration in mice on the expression of motoneuron β -endorphin and muscle acetylcholinesterase

M.E Smith, M.L. Amos, M.C. Lintern

Department of Physiology, Medical School, University of Birmingham, Birmingham B15 2TT, UK

There is considerable evidence that β -endorphin and its C-terminal dipeptide derivative, glycylglutamine, can control the expression of acetylcholinesterase (AChE) in cholinergic tissues, including skeletal muscle (Haynes et al., 1984; Haynes and Smith, 1985), heart (Nyquist-Battie and Millington, 1993), and sympathetic ganglion (Koelle, 1983). In previous work we have shown that repeated administration in mice of a low dose of pyridostigmine causes increases in the levels of AChE in skeletal muscle, after the acute inhibitory phase has worn off (Lintern et al., 1997; Lintern et al., 1997). We have also shown that this drug increases the expression of POMC-derived peptides, including β -endorphin, in motoneurons (Amos et al., 1996). Here we have compared the time course of the changes in AChE activity in the diaphragm muscle, and the expression of β -endorphin in motoneurons in the cervical spinal cord, following repeated administration of pyridostigmine, in the same animals.

In another study we showed that repeated treatment in mice with pyridostigmine sensitised the muscle to a subsequent low dose of the drug given 2 weeks after the repeated treatment was

discontinued (Lintern et al., 1997). Therefore the effect of a booster dose of pyridostigmine on both muscle AChE and motoneuron β -endorphin was also studied.

Pyridostigmine bromide (0.4 μ mol/kg) was administered to male BKW (6 month old) mice, twice a day for 3 weeks. In one group a further single low dose (0.4 μ mol/kg) of the drug was administered at 2 weeks after cessation of the repeated treatment, and the effects on the levels of AChE in the diaphragm muscle and the incidence of β -endorphin-immunoreactive motoneurons were compared with the effect of a single dose in animals which had had no previous treatment with the drug.

The molecular forms of AChE were separated on a sucrose density gradient as described before (Haynes et al., 1984) and the enzyme activity was determined using the method of Johnson and Russell (1975). Immunoreactivity for β -endorphin was detected in 20 μ m histological sections of cervical spinal cord using the immunoperoxidase method as described before (Hughes and Smith, 1994).

At 24 h after the end of the 3-week repeated treatment there was a down-regulation of total AChE activity and the activity of the major component molecular forms, including the functional A12 form, in the diaphragm, followed 1 week later by an up-regulation to levels significantly higher than normal (figure 1). The incidence of motoneurons in the cervical spinal cord which were immunoreactive for β -endorphin was extremely low in untreated mice but there was a significant increase in the incidence after the pyridostigmine treatment. However, the increase in β -endorphin immunoreactivity preceded the increase in AChE activity.

In the animals which had been given a further low dose of the drug at 2 weeks after the treatment the AChE activity had initially returned to normal, although the β -endorphin immunoreactivity was still significantly (over four-fold) elevated (figure 1). The effect of the 'booster' dose of the drug in these animals was compared with administration of the same dose of the drug to previously untreated animals. In the pretreated animals the booster dose caused a significant (approximately 130%) increase in the levels of the enzyme in the muscle at 3 h, but when the same single low dose of the drug was administered to untreated mice in which the levels of β -endorphin in the motoneurons was initially extremely low (figure 1, controls), the enzyme activity was unchanged at 3 h (although there was a three-fold increase in immunoreactive motoneurons at this time).

Thus after treatment with pyridostigmine when an increase in AChE levels in the muscle was seen, the increases were preceded by an increase in β -endorphin expression in the motoneurons. These findings are consistent with the increased expression of β -endorphin in the motoneuron being a prerequisite for the up-regulation of AChE in skeletal muscle. This work has been carried out with the support of CBD Porton Down. (c) British Crown copyright 1998/DERA. Published with permission of the Controller of Her Britannic Majesty's Stationary Office.

References

Amos ML, Smith ME, Ferry CB (1996). the effect of pyridostigmine bromide administration in mice on the expression of POMC-derived peptides in motoneurons. *J Physiol* 491P, 146P.

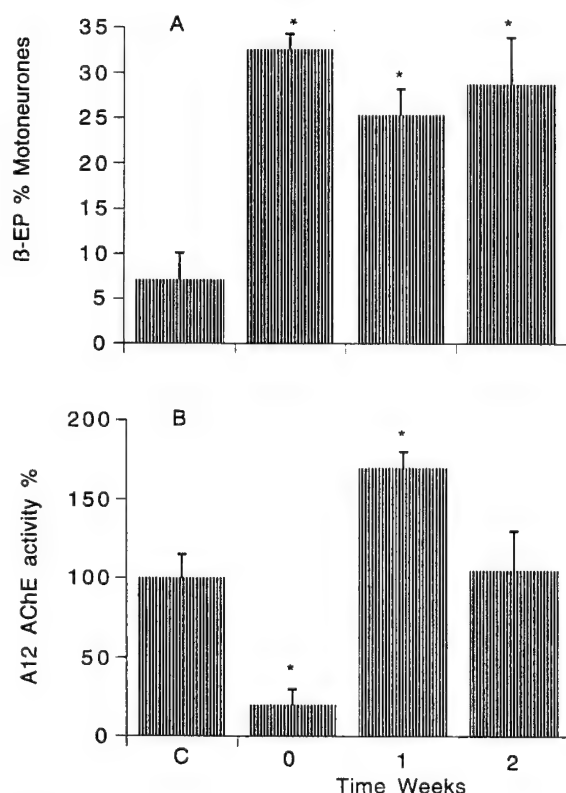


Figure 1. Time course of the changes in **A**, the proportion of β -endorphin immunoreactive motoneurone in cervical spinal cord, and **B**, functional A12 AChE activity in diaphragm muscle, at 24 h (0 weeks) and 1 and 2 weeks, after repeated treatment with pyridostigmine. *Significant compared to controls (C).

- Haynes LW, Smith ME (1985) Induction of endplate-specific acetylcholinesterase by b-endorphin C-terminal dipeptide in rat and chick muscle cells. *Biochem Soc Transac* 13, 174-175.
- Haynes LW, Smith ME, Smyth DG (1984) Evidence for the neurotrophic regulation of acetylcholinesterase in immature skeletal muscle by b-endorphin. *J Neurochem* 42, 1542-1551.
- Hughes S and Smith ME (1994). Upregulation of the pro-opiomelanocortin gene in motoneurons after nerve section in mice. *Mol Brain Res* 25, 41-49.
- Johnson CD, Russell RL (1975) A rapid and simple radiometric assay for cholinesterase, suitable for multiple determinations. *Analyt Biochem* 64, 229-238.

- Koelle GB, Ruch GA (1983) Demonstration of a neurotrophic factor for the maintenance of AChE and BuChE in the preganglionically denervated superior cervical ganglion of the cat. *Proc Nat Acad Sci* 80, 3106-3110.
- Lintern ML, Smith ME, Ferry CB (1997) Effects of pyridostigmine on acetylcholinesterase in different muscles of the mouse. *Human & Exp Toxicol*, 16, 18-24.
- Lintern ML, Smith ME, Ferry CB (1997) Effect of repeated treatment with pyridostigmine on acetylcholinesterase in mouse muscles. *Human Exp Toxicol*, 16, 158-165.
- Nyquist-Battie EC, Hagler KE, Millington WR (1993) Glycylglutamine regulates the expression of asymmetric acetylcholinesterase molecular forms in cultured cardiac postnatal myocytes *J Mol Cell Cardiol* 25, 1111-1128.

Metrifonate affects the peak interval performance in the rat

L. Spowart, B.H. Schmidt, F.J. van der Staay

Bayer CNS Research, Troponwerke GmbH, Neurather Ring 1, D-51063, Köln, Germany

Metrifonate is a member of the second generation of cholinesterase inhibitors (Giacobini, 1991) that has been found to be a promising therapeutic agent to combat Alzheimer's disease (Becker et al., 1990). In a number of studies metrifonate has been shown to improve the cognitive performance in various behavioural models (Schmidt et al., 1997). In this study the effect of metrifonate on timing behaviour was assessed. The rats were trained on a discrete-trial 20 s peak interval (PI) timing procedure in ten identical Skinnerboxes.

The PI procedure is a modification of the standard fixed interval procedure of reinforcement (Catania, 1970; Roberts, 1981). On a random half of the trials a lever is presented and the animal is free to respond at any time, but only the first lever-press res-

ponse after 20 s is reinforced with a 45 mg food pellet. On the remaining trials, the peak trials, no food is given and the lever remains available until 50 s have elapsed. Then the lever is retracted for 10 seconds before the next trial starts. On the peak trials, response rate of trained animals increases to a peak, then the response rate decreases.

The effects of a single administration of metrifonate (10 mg/kg and 30 mg/kg) on responding on a 20 s peak procedure were assessed in Harlan Wistar rats. The animals had received six daily sessions on this schedule prior to drug administration. Metrifonate was administered orally 30 min before the seventh session started. Data were collected for each rat only on peak trials and sessions lasted 30 min.

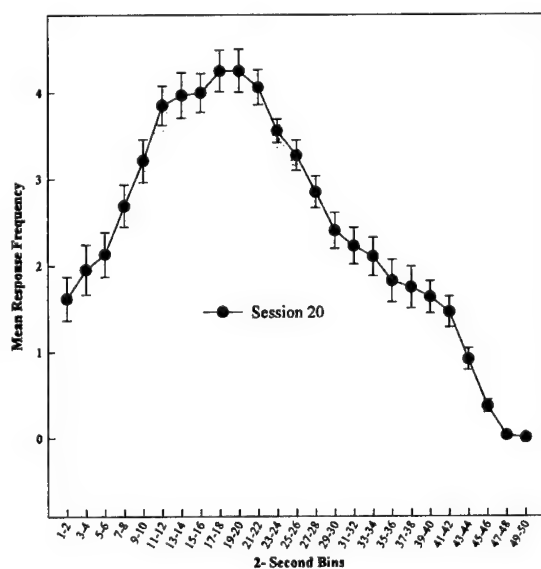


Figure 1. The effects of metrifonate in doses of 10 mg/kg, 30 mg/kg and vehicle (sodium citrate buffer) on response frequency during the 20 s peak procedure. Results are \pm S.E.M., $n = 9-10$ per group.

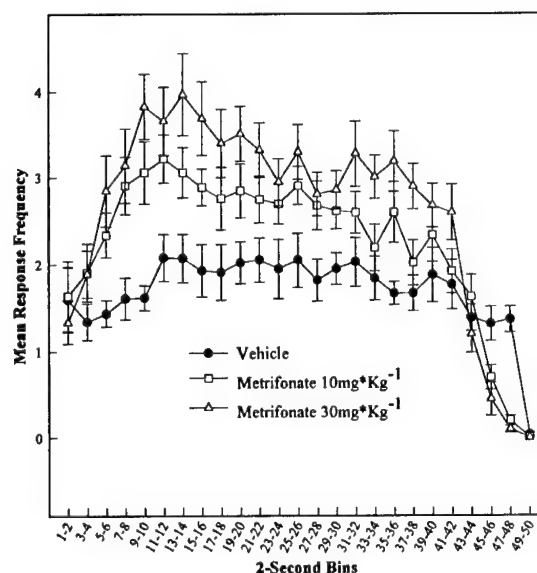


Figure 2. Mean response frequency \pm S.E.M. ($n = 30$) during the 20 s peak procedure at session 20.

Metrifonate treatment increased the response frequency, averaged over all bins (general mean: $F_{2, 26} = 8.98$, $P < 0.01$). Post hoc comparison (LSD) revealed that the response frequencies of both the groups of rats treated with 10 and 30 mg/kg metrifonate differed from the vehicle-treated group. The distribution of responses was also affected by the metrifonate treatment (treatment by BIN interaction: $F_{48, 624} = 3.11$, $P < 0.01$). Metrifonate-treated rats showed more of a peak, and this effect appeared to be dose-dependent.

The effects seen by acute metrifonate were achieved when the animals had not yet reached optimal level of performance (i.e., the maximum response rate was not at 20 s).

Training continued to demonstrate that the animals could reach an optimal level of performance. The animals achieved this after 20 sessions (see figure 2), the maximum response rate being at 19–20 s.

The behaviour of a rat in time discrimination procedures, such as the peak procedure is thought to be understood in the terms of an information processing model, which is to measure, compare and store the expected time of reinforcement. Shifts in the peak functions are thought to be used as evidence in the remembered time of reinforcement (Meck and Church, 1988). The present study suggests that metrifonate might improve acquisition of the timing task. Further study is required with subchronic metrifonate to determine whether metrifonate would also facilitate the rate of acquisition of the timing task in analogy with the previously reported improved acquisition of eye-blink conditioning in aging rabbits (Kronforst-Collins et al., 1997).

References

- Becker RE, Colliver J, Elble R, Feldman E, Giacobini E, Kumar V, Markwell S, Moriearty P, Parks R, Shillcut S D, Unni, L, Vicari, E, Womack C, Zec, RF (1990). Effects of metrifonate, a long lasting cholinesterase inhibitor, in Alzheimer's disease: Report of an open trial. *Drug Dev Res* 19, 425–434.
- Catania AC (1970). Reinforcement schedules and psychophysical judgements. A study of some temporal properties of behaviour. In Schoenfeld (Ed). *The theory of reinforcement schedules*, 1–42.
- Giacobini E (1991). The second generation of cholinesterase inhibitors: pharmacological aspects. In Becker R, Giacobini E. (Eds). *Cholinergic Basis for Alzheimer's therapy*, New York, Birkhäuser, 247–262.
- Kronforst-Collins MA, Moriearty PL, Ralph M, Becker RE, Schmidt B, Thompson LT, Disterhoft J F (1997). Metrifonate treatment enhances acquisition of eyeblink conditioning in aging rabbits. *Pharmacol Biochem Behav*, 56 (1) 103–11.
- Meck WH, Church RM. (1988) Biological basis of the remembered time of reinforcement. In Commons ML, Church RM, Stellar JR, Wagner AR (Eds) *Quantitative Analyses of Behaviour: Biological Determinants of Reinforcement*, Vol. 7, Erlbaum, Hillsdale, N J, 121–138.
- Roberts S (1981). Isolation of the Internal Clock. *Animal Behavioural Processes*. *J Exp Psychology* 7, 242–268.
- Schmidt BH, Hinz VC, van der Staay FJ. (1997). Cognition enhancement by metrifonate: Evidence from animal studies. In Iqbal K, Winblad T, Nishimura T, Takeda HM, Wisniewski (Eds.), *Alzheimer's disease: From Molecular Biology to Therapy*, John Wiley & Sons Ltd, Chichester, 781–786.

102

Cholinergic/monoaminergic markers in the brain, and behavioral effects of cholinergic and serotonergic drugs in aged rats with cognitive deficits

J. Stemmelin, C. Kelche, C. Lazarus, J.-C. Cassel

LN2C, UMR 7521 ULP/CNRS, 12, rue Goethe, F-67000 Strasbourg, France

Compared to young adult rats, rats aged of 20 months or more show a more or less pronounced impairment in a variety of learning/memory tests (e.g., Ingram et al., 1994). Numerous studies suggest that cholinergic dysfunctions might be involved in such deficits (e.g., Collerton, 1986). In the present study, 3-month- and 24-month-old Long Evans female rats were used. In a water maze task, we first assessed spatial reference memory deficits (acquisition and probe trial) and constituted a population of aged rats showing moderate to severe impairment. Using HPLC detection, we then determined the concentration of acetylcholine (ACh), nor-epinephrine (NE), serotonin (5-HT) and a few metabolites (DOPAC, HVA and 5-HIAA), in the striatum, the hippocampus, the frontal, occipital and entorhinal cortex after killing by microwave irradiation (1.4 s, 4.5 kW). Finally, a separate sample of rats was tested in a water maze for working memory performance in order to assess the effects of the muscarinic antagonist scopolamine (0.2 and 0.5 mg/kg, i.p.), the muscarinic agonist pilocarpine (0.5 mg/kg, i.p.), the 5-HT_{1A} agonist 8-OH-DPAT (0.1, 0.3 and 1 mg/kg, i.p.), the α_1 antagonist prazosin (1 mg/kg, i.p.) and the 5-HT_{1A} antagonist NAN 190 (2 mg/kg, i.p.). Drugs were also used in combination (e.g., scopolamine or NAN 190 with pilocarpine).

On the 2nd, 3rd, 4th and last testing days of the reference memory task, the average distance to reach the platform was

significantly higher in aged as compared to young rats (data not shown). In the probe trial, young rats spent a significantly longer time in the platform-quadrant than aged rats (25.8 ± 1.7 s and 18.3 ± 1.1 s, respectively, $P < 0.001$). The neurochemical results for which significant differences were found are shown in table I. In aged rats, significant correlations could be found between water maze performance (probe trial) and: ACh in the striatum ($r = 0.45$, $P = 0.05$), 5-HT in the dorsal hippocampus ($r = 0.53$, $P = 0.02$), 5-HIAA in the frontal cortex, dorsal hippocampus and striatum ($r = 0.47$ at least, $P = 0.039$ at most), 5-HIAA/5-HT ratios in the striatum and frontal cortex ($r = 0.46$, $P = 0.046$ and $r = 0.51$, $P = 0.034$, respectively), and NE in the dorsal hippocampus ($r = 0.57$, $P = 0.016$).

The psychopharmacological data are shown in table II. Briefly, 0.1 mg/kg scopolamine HBr, 0.5 mg/kg MBr, 0.5 mg/kg pilocarpine and 0.1 mg/kg 8-OH-DPAT induced no significant modification of performances. At 0.5 mg/kg, scopolamine HBr produced a significant deficit in young and aged rats, but the deficit found in young rats under scopolamine treatment did not exceed that found in aged rats given saline injections. NAN 190 and 0.3 mg/kg 8-OH-DPAT worsened performances, but only in aged rats. At 1.0 mg/kg, 8-OH-DPAT impaired performances in young and aged rats. Prazosin improved performances in young and

Table I. Neurochemical markers (values are ng/mg microwave-irradiated tissue).

Marker	Brain region	Young	Aged
ACh	Striatum	2.78 ± 0.40	1.72 ± 0.07
DA	Dorsal hippocampus	0.72 ± 0.09	0.39 ± 0.04
5-HT	Striatum	3.88 ± 0.35	3.10 ± 0.12
	Occipital cortex	2.04 ± 0.21	1.64 ± 0.08
5-HIAA/5-HT	Frontal cortex	0.68 ± 0.02	1.03 ± 0.09
	Dorsal hippocampus	1.03 ± 0.05	1.65 ± 0.17
	Ventral hippocampus	0.92 ± 0.03	1.51 ± 0.16
	Entorhinal cortex	0.45 ± 0.05	0.73 ± 0.08
HVA	Striatum	3.37 ± 0.16	2.61 ± 0.14
NE	Striatum	1.15 ± 0.12	0.84 ± 0.04
	Dorsal hippocampus	1.80 ± 0.12	1.40 ± 0.08
	Ventral hippocampus	1.82 ± 0.09	1.43 ± 0.06

Table II. Distances (in cm ± S.E.M.) to reach the platform in the working memory task (four daily trials with a new location of the platform each day) under various drug treatments (doses given in parentheses are in mg/kg, i.p.).

Drug condition	Young (n = 11)	Aged (n = 41)
Saline 0.9%	208.5 ± 19.1	413.1 ± 26.3
Scopolamine MBr (0.5)	206.6 ± 32.2	306.8 ± 35.9
Scopolamine HBr (0.2)	268.4 ± 36.8	451.2 ± 37.4
Scopolamine HBr (0.5)	394.0 ± 70.9	706.4 ± 39.1
Pilocarpine (0.5)	188.8 ± 16.1	350.8 ± 34.9
NAN 190 (2.0)	253.5 ± 11.8	638.3 ± 38.0
8-OH-DPAT (0.1)	178.3 ± 26.2	409.5 ± 33.0
8-OH-DPAT (0.3)	251.1 ± 31.0	519.3 ± 34.0
8-OH-DPAT (1.0)	496.3 ± 78.8	747.2 ± 44.3
Prazosin (1.0)	158.9 ± 8.2	315.8 ± 24.0
Scopolamine (0.5) + Pilocarpine (0.5)	375.6 ± 41.1	561.6 ± 29.5
Scopolamine (0.5) + NAN 190 (2)	634.7 ± 76.7	730.1 ± 38.0
Scopolamine (0.2) + 8-OH-DPAT (0.1)	410.3 ± 68.1	664.2 ± 36.9
Pilocarpine (0.5) + NAN 190 (2)	252.8 ± 31.1	381.4 ± 27.6

aged rats. Pilocarpine attenuated the scopolamine-induced impairments, but only in aged rats. In young rats, NAN 190 potentiated the scopolamine-induced deficits. In both groups, the combination of 8-OH-DPAT and scopolamine at ineffective doses (0.1 and 0.2 mg/kg, respectively) induced clear-cut impairments which were more pronounced in aged than in young rats. The combination of pilocarpine and NAN 190 had no significant effect.

In conclusion of these experiments, we confirm that aging results in (variable degrees of) cognitive impairment. Although the water maze task is particularly sensitive to disruption of spatial memory processes and despite a crucial involvement of the hippocampus in such mnemonic processes, but also of cholinergic function in learning and memory, we failed to find any significant correlation between water maze performances and cholinergic (but also other) markers in the hippocampus (but also cortical regions). The only significant

correlation found between memory performances and ACh concentration was in the striatum, a finding suggesting that the age-related cholinergic changes in the striatum might play an important role in the behavioral deficits observed. From our psychopharmacological data it is also clear that the cognitive deficits observed are not an exclusive matter of cholinergic dysfunction: injections of NAN 190 and 8-OH-DPAT were more efficient in aged as compared to young rats, as was the case for combined injections of scopolamine and 8-OH-DPAT at subamnesic doses.

References

- Collerton D (1986) Cholinergic function and intellectual decline in Alzheimer's disease. *Neuroscience* 19, 1-28.
- Ingram DK, Jucker M, Spangler EL (1994) Behavioral manifestations of aging. *Pathobiol. Aging Rat* 2, 149-170.

103

Human acetylcholinesterase transgenic mice show elevated muscle expression and brain specific silencing

M. Sternfeld^a, J.D. Patrick^b, H. Soreq^a

^aDept. of Biological Chemistry, The Institute of Life Sciences, The Hebrew University of Jerusalem, 91904 Israel
^bDivision of Neuroscience, Baylor College of Medicine, Houston, TX 77030-3498, USA

To study the regulation of AChE expression in the mammalian brain and muscle, novel transgenic FVB/N mouse lines were created with several variants of human AChE. The human ACHEcDNA constructs that were employed for this study were CMV I4-ACHE, encoding for the 'readthrough' AChE isoform C-terminated with the pseudointron I4-derived hydrophilic peptide (Karpel et al., 1996) and CMV InE6-ACHE which codes for the insertion-inactivated isoform C-terminated with the exon 6-derived peptide but with no capacity to hydrolyze ACh (Sternfeld et al., 1998). The properties of these transgenic lines were compared to those of the human promoter (Hp) E6-AChE transgenic line which overexpresses the brain and muscle-abundant isoform carrying the exon 6-derived amphipathic C-terminal peptide (Beeri et al., 1995) and of control FVB/N mice. Each of these lines displayed unimpaired Mendelian inheritance of the transgene for over 5 generations, showing that it is not lethal. Kinetic followup of PCR amplifications reflecting copy number of the transgene revealed similar copy number for the two independent pedigrees with CMV I4-AChE transgenes and a different copy number for the line with HpE6-AChE and CMV InE6-AChE line. The efficacy of AChE expression in brain and muscle tissues from these transgenic mice was evaluated by ATCh hydrolysis rates in tissue homogenates in the presence and absence of the specific BChE inhibitor IsoOMPA. These measurements demonstrated drastic differences in the specific activity of muscle AChE. While InE6-AChE transgenics revealed activities 1.6-fold higher than controls, the two I4 AChE lines displayed muscle AChE activities 25- and 350-fold of control mice. In contrast to the drastic variabilities in muscle AChE levels, brain AChE specific activities remained grossly similar in all of these transgenic lines. These ranged from 95% of control activity for InE6-AChE transgenics to 140% and 131% of control for the two I4 AChE transgenic lines. HpE6-AChE transgenics revealed the highest values, reaching activities 1.5-fold higher than the brain activity in control FVB/N mice (Beeri et al., 1995). These results demonstrate considerable permissivity of muscle AChE expression variations but maintainance of brain AChE levels within a very narrow activity window. The finding that two mouse pedigrees carrying apparently similar copy numbers of the I4 AChE transgene under control of the CMV

promoter show such different levels of AChE overexpression may reflect position effects of the insertion of these transgenes into the host mouse genome. This overexpression apparently does not interfere with neuromuscular properties, which is compatible with the non-synaptic localization of I4 AChE as well as with its minimal effect on neuromuscular junction development (Seidman et al., 1995; Sternfeld et al., 1998). Within the same mouse pedigrees with massive muscle AChE increases, variations in brain AChE activity were remarkably limited. It is likely that the viability of these transgenic pedigrees depended on minimal changes in their brain AChE levels, so that the only lineages that survived were those where the transgene's expression in the brain was effectively silenced. This suggests that the upstream domains adjacent to the two I4 AChE transgenes should include effective brain silencing in addition to muscle enhancing elements. These newly created transgenic models should therefore be most valuable for testing the biological effects of AChE overproduction and sustained inhibition and for exploring the molecular mechanisms controlling both the expression patterns of these transgenes and the ability of the host mice to accommodate these changes.

References

- Beeri R., Andres C., Lev-Lehman E., Timberg R., Huberman T., Shani M., Soreq, H. (1995). Transgenic expression of human acetylcholinesterase induces progressive cognitive deterioration in mice. *Curr. Biol.*, 5, 1063-1071.
- Karpel R., Sternfeld M., Ginzberg D., Guhl E., Graessman A., Soreq H. (1996). Overexpression of alternative human acetylcholinesterase forms modulates process extensions in cultured glioma cells. *J. Neurochem.*, 66, 114-123.
- Seidman S., Sternfeld M., Ben Aziz-Aloya R., Timberg R., Kaufer-Nachum D., Soreq, H. (1995). Synaptic and epidermal accumulations of human acetylcholinesterase is encoded by alternative 3'-terminal exons. *Mol. Cell. Biol.* 15, 2993-3002.
- Sternfeld M., Ming G-L., Song H-J., Sela K., Poo M-M., Soreq H. (1998). Acetylcholinesterase enhances neurite growth and synapse development through alternate contributions of its hydrolytic capacity, core protein and variable C-termini. *J. Neurosci.*, 18, 1240-1249.

104

Role of Ca²⁺ and NO in differential effects of extra and intracellular aluminum on acetyl-CoA and acetylcholine metabolism in rat brain nerve terminals

A. Szutowicz, H. Bielarczyk, M. Tomaszewicz, A. Jankowska

Department of Clinical Biochemistry, Medical University of Gdańsk, Dębinki 7, 80-211 Gdańsk, Poland

Excessive accumulation of aluminum (Al) and increased NO synthesis may coexist in brains affected by Alzheimer's and Parkinson's diseases, dialysis encephalopathy and by many other neurodegenerative brain diseases (Davson and Davson 1996; Mei-

ri et al., 1993). Bulk Al concentrations in diseased brains were reported to be within 0.1-0.5 mM, whereas its intracellular level was likely to reach 10 mM range. Al forms slowly dissociating complexes with several extra- and intracellular sites that function

dependent on binding with Ca. Thereby, Al may interfere with several Ca-dependent steps of cellular metabolism. Also, extensive NO production was found to disrupt Ca^{2+} homeostasis in neurons (Brorson et al., 1997). Our studies have shown that Al inhibited, and NO activated acetyl-CoA and acetylcholine (ACh) metabolism in nerve terminals (Bielarczyk et al., 1998; Tomaszewicz et al., 1997). Therefore, in this work we aimed to find out how simultaneous action of both pathogens may affect metabolism of acetyl moiety of ACh in this neuronal compartment and if it may be linked with changes in Ca^{2+} homeostasis.

Synaptosomes isolated from rat brains were incubated in depolarizing medium (30 mM KCl) while mitochondria in sucrose-potassium medium using 2.5 mM pyruvate and 2.5 mM L-malate as energy substrates for 30 and 15 min, respectively (Bielarczyk et al., 1998). In such conditions, Al did not pass neuronal plasma membrane (Shi, Haug, 1990). In Ca-free medium 0.25 mM Al inhibited pyruvate utilization (28%), decreased acetyl-CoA content in synaptoplasm (25%), increased resting ACh release (50%) but did not change total content intrasynaptosomal Ca, which was equal to about 3 nmol/mg of protein. In the same conditions 0.2 mM sodium nitroprusside (SNP) had no effect on these metabolic parameters. Hence, extracellular Al itself may exert some Ca-like effects increasing nonquantal ACh release from terminals while NO apparently had no such capacity due to the lack of Ca.

Addition of 1 mM Ca increased intrasynaptosomal Ca content from 3.3 to 33 nmol/mg of protein and caused 40% inhibition of pyruvate utilization but simultaneously increased synaptoplasmic acetyl-CoA level (50%) and ACh release from synaptosomes (170%). In these conditions 0.25 mM Al decreased bulk Ca level in synaptosomes (40%) with no change in pyruvate utilization (not shown). Accordingly, it decreased synaptoplasmic acetyl-CoA (29%) and inhibited overall and Ca-evoked ACh release by 39 and 80%, respectively (figure 1). On the other hand, 0.25 mM SNP increased synaptoplasmic acetyl-CoA (51%) as well as total and Ca-evoked ACh release (about 40%) (figure 1). Al reversed SNP effects bringing synaptoplasmic acetyl-CoA and ACh release back to control values. Al-evoked decrease of synaptosomal Ca was likely to result from inhibition of voltage-dependent, verapamil sensitive Ca channels (Bielarczyk et al., 1998). It would inhibit Ca-dependent direct transport of acetyl-CoA from mitochondria yielding inhibition of

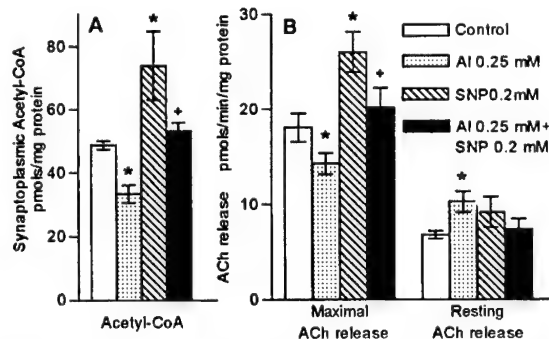


Figure 1. A. Effect of Al and SNP on acetyl-CoA content in synaptoplasm of depolarized synaptosomes incubated at 1 mM [Ca]. B. ACh release from depolarized synaptosomes incubated in the absence (resting release) and in the presence of 1 mM Ca (maximal release). Ca-evoked release was defined as a difference between respective maximal and resting ACh releases (not shown). Results are means \pm SEM of five to 15 experiments performed in duplicate. * $P < 0.05$, compared with respective control; * $P < 0.05$, compared with respective 0.25 mM Al. All maximal ACh release values were different from respective resting release values.

ACh synthesis as well as directly by suppression of its quantal release. On the other hand, impairment of energy metabolism by NO led to the rise of synaptoplasmic Ca which activated efflux of acetyl-CoA from mitochondria and increased its utilization for ACh synthesis and subsequent release (Brorson et al., 1997; Tomaszewicz et al., 1997). Inhibition of energy production by NO along with increased utilization of acetyl-CoA for ACh synthesis in cholinergic synaptosomes can make them particularly susceptible to deleterious effects of this compound. Reversal NO effects by extracellular Al is in accord with reciprocal action of both compounds in changes of Ca homeostasis in nerve terminals (Bielarczyk et al., 1998; Brorson et al., 1997).

Isolated mitochondria were used to study possible effects of intracellular Al. It has been found that Al may be transported through the plasma membrane as Al-transferrin complex (Shi and Haug, 1990). In Ca-free medium containing 5 mM Pi and 0.02 mM EDTA, 0.5 mM Al decreased pyruvate utilization (33%, not shown) and acetyl-CoA content (55%) but did not affect total CoA level. Addition of 0.01 mM Ca suppressed pyruvate utilization (36%) as well as acetyl-CoA (29%) and CoA (10%) contents (figure 2). In these conditions Al increased $[\text{Ca}^{2+}]$ in the medium from 0.3 μM to 6.8 μM and intramitochondrial Ca content from 21 to 29 nmol/mg of protein along with inhibition of pyruvate utilization (19%), decrease of acetyl-CoA (38%) and no change in CoA level. Addition of 0.2 mM SNP caused inhibition of pyruvate utilization as well as depression of acetyl-CoA (about 40%) and increase of CoA (about 50%) content irrespective of Ca presence in the medium (figure 2). These data indicate that cytoplasmic Al itself, unlike Ca, did not increase permeability of the mitochondrial membrane to acetyl-CoA/CoA. However, it was likely to increase cytoplasmic $[\text{Ca}^{2+}]$ due to competition with Ca-binding sites. This phenomenon together with inhibition by Al of Na/Ca antiporter might lead to overloading mitochondria with Ca and loss of CoA derivatives from this compartment. On the contrary, effects of NO on mitochondrial acetyl-CoA/CoA seem to be Ca-independent, although NO was also able to increase $[\text{Ca}^{2+}]$ in neuronal cytoplasm (Brorson et al., 1997).

Hence, in brain in vivo, extracellular Al is likely to exert Ca/NO-antagonistic effects on acetyl-CoA and ACh metabolism in nerve terminals, whereas intracellular Al would exacerbate symptoms of Ca and NO toxicity due its ability to increase $[\text{Ca}^{2+}]$ in neuronal cytoplasm. Therefore, final effect of Al accumulation on acetyl-CoA and ACh metabolism in the brain may depend on both, absolute Al concentrations and its ratios in extra and intracellular compartments. This work was supported by K.B.N project 6 PO4A 013 10.

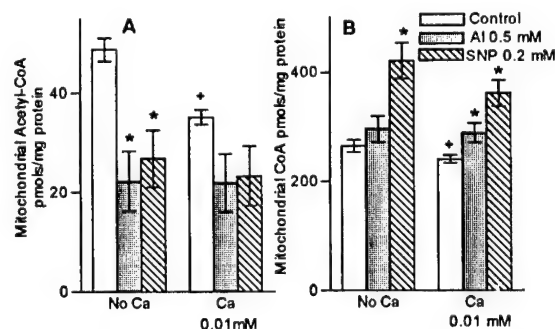


Figure 2. Effect of Al and SNP on acetyl-CoA (A) and CoA contents (B) in whole brain mitochondria. Results are means \pm S.E.M. of five to 15 experiments performed in duplicate. * $P < 0.05$, compared with respective control; * $P < 0.05$, compared with respective no Ca conditions.

References

- Bielarczyk H, Tomaszewicz M, Szutowicz A (1998) Effect of aluminum on acetyl-CoA and acetylcholine metabolism in nerve terminals. *J. Neurochem.* 70, 1175–1181.
- Brorson JR, Sulist RA, Zang H (1997) Nitric oxide disrupts Ca^{2+} homeostasis in hippocampal neurons. *J. Neurochem.* 68, 95–105.
- Davson VL, Davson TM (1996) Nitric oxide in neuronal degeneration. *Proc. Soc. Exp. Biol. Med.* 212, 33–40.
- Meiri H, Banin E, Roll M, Rousseau (1993) Toxic effects of aluminum on nerve cells and synaptic transmission. *Prog. Neurobiol.* 40, 89–121.
- Shi B, Haug A (1990) Aluminum uptake by neuroblastoma cells. *J. Neurochem.* 55, 551–558.
- Tomaszewicz M, Bielarczyk H, Jankowska A, Szutowicz A (1997) Modification by nitric oxide acetyl-CoA and acetylcholine metabolism in nerve terminals. In: *Neurochemistry: Cellular, Molecular and Clinical Aspects* (Telken AW, Korf J eds) pp. 993–997, Plenum Publishing, London.

105

Acetylcholinesterase of *S. mansoni* (Trematode). Interaction of globular species with glycosaminoglycans

R. Tarrab-Hazdai^a, I. Silman^b, L. Toker^b, D. Schechtman^a, R. Arnon^a

The Weizmann Institute of Science, Departments of ^aImmunology and ^bNeurobiology, Rehovot 76100, Israel

Vertebrate acetylcholinesterase (AChE, EC 3.1.1.7) occurs in multiple molecular forms, both asymmetric (A) and globular (G) (Massoulié et al., 1993). In invertebrates, the diversity of AChE molecular forms is more limited than in vertebrates (Massoulié et al., 1993; Talesa et al., 1995) and seems to be restricted to G forms. The existence of several molecular forms reflects primarily differences in their mode of attachment at the cell surface. The A forms of AChE have been reported to interact with heparin, a sulfated glycosaminoglycan (Brandan et al., 1984), and this interaction is responsible for their attachment to the basal lamina within the synaptic cleft (Brandan et al., 1986). However, several studies have reported interaction of G forms with heparin, although less strongly than the A forms (Ramirez et al., 1990; Sine et al., 1994; Talesa et al., 1995), and it has been suggested that they may also be associated with the basal lamina.

In this study the interaction of AChE (EC 3.1.1.7) and heparin was studied by affinity chromatography. In the parasite, *S. mansoni*, AChE is present throughout the life cycle (Arnon, 1991). As in vertebrates, it may play a role in termination of synaptic transmission at cholinergic synapses (Barker et al., 1966), but is also present on the tegument, where its biological role remains to be established (Tarrab-Hazdai et al., 1984; Camacho et al., 1994). At early stages of the life cycle, viz. in the cercaria and schistosomula, AChE appears as two principal molecular forms, both G forms, in approximately equal amounts, with sedimentation constants of 6.5 and 8 S (Espinoza et al., 1988; Camacho et al., 1994). The 6.5 S form, which is amphiphilic, is solubilized by bacterial phosphatidylinositol-specific phospholipase C (Espinoza et al., 1988, 1991), and, most likely, is homologous to the GPI-anchored G_2 form found in *Torpedo* electric organ and elsewhere (Silman and Futerman, 1987). Both forms are fully solubilized by the non-ionic detergent, Triton X-100. Upon passing such a detergent extract over a heparin-agarose column, only the 8 S form was retained on the column (figure 1). The bound AChE could be progressively eluted by increasing the salt concentration, ca. 0.5 M NaCl being needed for complete elution. Monoclonal antibodies, raised against *S. mansoni* AChE (Espinoza et al., 1995), also distinguished between the two forms. Thus mAb SA7 bound the 6.5 S form selectively, whereas mAb SA57 recognized the 8 S form. Selective inhibition experiments, carried out on live parasites with the covalent AChE inhibitor, echothiophate (phospholine), which does not penetrate the tegument, selectively inhibited the 6.5 S form, thus suggesting an internal location for the 8 S form (Camacho et al., 1994). The selective binding of

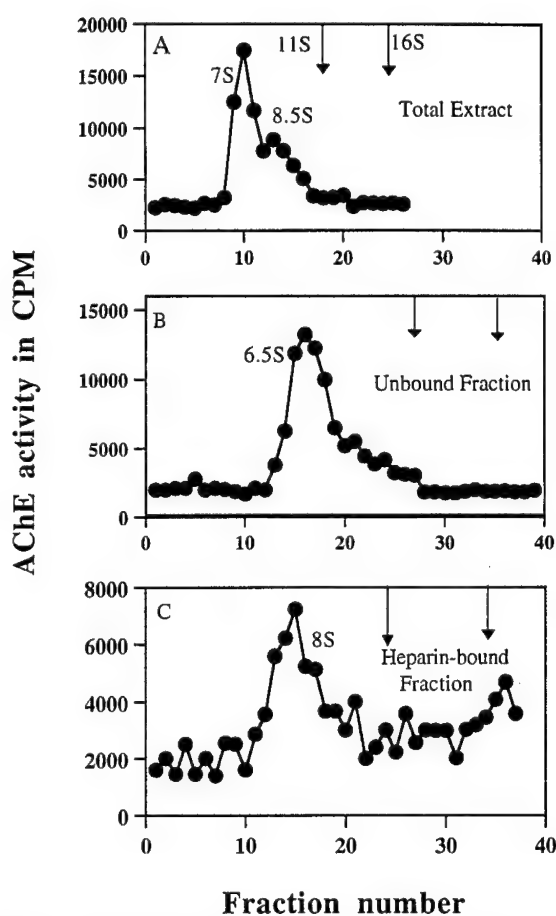


Figure 1. Differential binding of molecular forms of AChE from *S. mansoni* to an heparin-agarose column. A total extract of cercaria in 1% Triton X-100/0.05 M Tris hydrochloride, pH 7.5, was applied to a heparin-agarose column. Bound AChE activity was eluted with a 0–1 M NaCl gradient in the same buffer containing 0.1% Triton X-100 and pooled. Samples were analyzed by sucrose gradient centrifugation on 5–20% sucrose containing 0.1% Triton X-100/0.05 M Tris hydrochloride, pH 7.5. **A)** Total extract; **B)** Unbound fraction; **C)** Heparin-bound fraction. The arrows denote the positions of the markers: catalase (11 S) and β -galactosidase (16 S).

the 8 S form to heparin suggests that, within the parasite, it may be associated with the extracellular matrix at cholinergic synapses.

References

- Arnon R (199) Immuno-parasitological parameters in schistosomiasis—a perspective view of a vaccine-oriented immunochemist. *Vaccine* 6, 379–394
- Barker LR, Bueding E, Timms AR (1966) The possible role of acetylcholine in *Schistosoma mansoni*. *Br J Pharmacol* 26, 656–665
- Brandan E, Inestrosa NC (1984) Binding of asymmetric forms of acetylcholinesterase to heparin. *Biochem J* 221, 415–422
- Brandan E, Inestrosa NC (1986) The synaptic form of acetylcholinesterase binds to cell-surface heparan sulfate proteoglycans. *J Neurosci Res* 15, 185–196
- Camacho M, Tarrab-Hazdai R, Espinoza B, Arnon R, Agnew A (1994) The amount of acetylcholinesterase on the parasite surface reflects the differential sensitivity of schistosoma species to metrifonate. *Parasitology* 108, 153–160
- Espinoza B, Tarrab-Hazdai R, Silman I, Arnon R (1988) Acetylcholinesterase of *S. mansoni* is anchored to the membrane via covalently attached phosphatidylinositol. *Mol Biochem Parasitol* 29, 171–179
- Espinoza B, Silman I, Arnon R, Tarrab-Hazdai R (1991) Phosphatidylinositol-specific phospholipase C induces biosynthesis of acetylcholinesterase via diacylglycerol in *S. mansoni*. *Eur J Biochem* 195, 863–870
- Espinoza B, Parizade M, Ortega E, Tarrab-Hazdai R, Zilberg D, Arnon R (1995) Monoclonal antibodies against acetylcholinesterase of *S. mansoni*. Production and characterization. *Hybridoma* 14, 577–586
- Massoulié J, Pezzementi L, Bon S, Krejci E, Vallette F-M (1993) Molecular and cellular biology of cholinesterases. *Prog Neurobiol* 41, 31–91
- Ramirez G, Barat A, Fernandez H (1990) Interaction of asymmetric and globular acetylcholinesterase species with glycosaminoglycans. *J Neurochem* 54, 1761–1768
- Silman I, Futerman, AH (1987) Modes of attachment of acetylcholinesterase to the surface membrane. *Eur J Biochem* 170, 11–22.
- Sine JP, Toutant JP, Colas B (1994) Butyrylcholinesterase amphiphilic forms of the mucosal cells of rat intestine bind heparin. *Biochem Biophys Res Comm* 201, 1376–1381
- Talesa V, Grauso M, Giovannini GR, Rosi G, Toutant JP (1995) Acetylcholinesterase in tentacles of *Octopus Vulgaris* (Cephalopoda). Histochemical localization and characterization of specific high salt-soluble and heparin-soluble fraction of globular forms. *Neurochem Int* 27, 201–211
- Tarrab-Hazdai R, Levi-Schaffer F, Smolarsky M, Arnon R (1984) Acetylcholinesterase of *Schistosoma mansoni*: Antigenic cross-reactivity with *Electrophorus electricus* acetylcholinesterase and its functional implications. *Eur J Immunol* 14, 205–209.

106

A brain slice model of seizure activity induced by an organophosphorus anticholinesterase

J.E.H. Tattersall, S.J. Wood

Biomedical Sciences Department, CBD Porton Down, Salisbury, Wiltshire, SP4 0JQ, UK

We are using an in vitro brain slice model to investigate the mechanisms of seizure activity induced by the organophosphorus anticholinesterase soman and to evaluate the effectiveness of potential anticonvulsant drugs against these seizures. Unlike previously-reported slice studies with nerve agents (Boyer et al., 1989; Lebeda and Rutecki, 1985; Sarvey and Williamson, 1985a; Williamson and Sarvey, 1985), this model contains the entorhinal cortex as well as the hippocampus. This allows the study of the spread of seizure discharges within the limbic system, and the development of prolonged, sustained discharges that are rarely seen in the simple hippocampal slice preparation (Nagao et al., 1996).

Male Dunkin-Hartley guinea pigs (180–240 g) were anaesthetised with halothane and killed by decapitation. The brain was removed and 400 µm thick horizontal slices were cut from a block of tissue containing the hippocampus and entorhinal cortex using a Vibratome. Experiments were performed in a Haas type interface chamber. The tissue was perfused with artificial cerebral spinal fluid (ACSF, composition in mM: NaCl 125; KCl 5; MgCl₂ 1; NaH₂PO₄ 1.25; NaHCO₃ 26; CaCl₂ 2; glucose 10; pH 7.4, gassed with 95% O₂–5% CO₂) and maintained at 34 °C. Extracellular field potential responses were recorded using glass microelectrodes filled with 2 M NaCl (2–10 MΩ resistance).

Perfusion with soman (1 µM) induced a second population spike in the evoked field potential in CA1 or CA3 within 15–20 min. In almost all slices tested, this developed into spontaneous seizure activity within 30–40 min of soman application. As well as interictal bursts, many slices also showed longer periods of high frequency bursting analogous to ictal seizure activity (figure 1). This activity appeared similar to that induced by the muscarinic agonist pilocarpine in this preparation (Nagao et al., 1996).

Both the second population spike and the spontaneous discharges could be blocked by diazepam, as previously reported (Boyer et

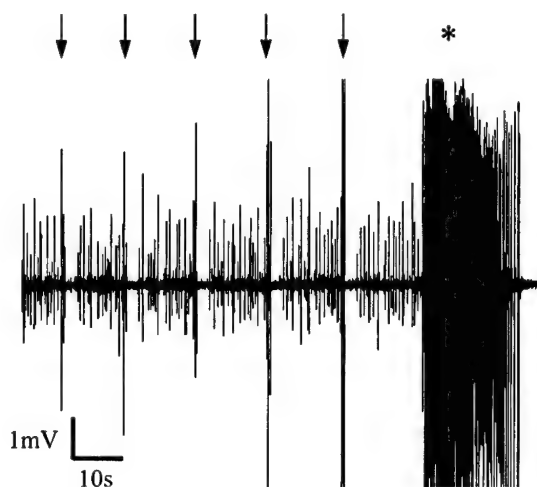


Figure 1. Epileptiform activity recorded in CA1 of a slice perfused with soman (1 µM). As well as simple interictal-like bursts, many slices also showed longer periods (1 s or more) of high frequency bursting (arrows), each followed by a period of inhibition of the simple discharges. This figure also shows a long-lasting episode (nearly 20 s) of high frequency bursting (asterisk), analogous to ictal seizure activity, which was followed by a silent period of 10s. The sustained activity was not seen in CA1 when a cut was made between CA3 and the entorhinal cortex (not shown).

al., 1989; Sarvey and Williamson, 1985; Williamson and Sarvey, 1985b), and by the AMPA/kainate antagonists CNQX and DNQX, but not by the non-competitive NMDA antagonist MK-801. We are now testing the effects of other anticonvulsant drugs on soman-induced epileptiform activity in this preparation. Supported by the Ministry of Defence. (c) British Crown Copyright 1998 / DERA. Published with the permission of the Controller of Her Britannic Majesty's Stationery Office.

References

- Boyer CE, Eslin DE, Juliano SL and Sarvey JM (1989) *Proc Med Def Biosci Rev* 119–122
 Lebeda FJ and Rutecki PA (1985) *Proc Med Def Biosci Rev* 503–524
 Nagao T, Alonso A and Avoli M (1996) *Neuroscience* 72, 399–408
 Sarvey, J.M. and Williamson A.M., *Proc Med Def Biosci Rev* (1985) 421–423
 Williamson AM and Sarvey JM (1985a) *J Pharmacol Exp Ther* 235, 448–455
 Williamson AM and Sarvey JM (1985b) *Soc Neurosci Abstr* 11, 596

107

His 186 requires the presence of the α 192–193 disulfide bridge to be able to interact with α -bungarotoxin

F.D. Testai, G.D. Venera, C. Peña, M. Biscoglio de Jiménez Bonino

Department of Biological Chemistry, Biological Chemistry and Physicochemistry Institute, School of Pharmacy and Biochemistry, University of Buenos Aires, Junín 956, (1113) Buenos Aires, Argentina

Multiple binding sites participate in the interaction between the nicotinic acetylcholine receptor (nAChR) and α -bungarotoxin (α -BgTx) (Conti-Tronconi et al., 1994).

We have previously reported the involvement of two histidine residues in such interaction (Lacorazza et al., 1992, 1996a, 1996b) and that His 134, which belongs to the cysteinyl loop α 124–127, being one of them (Venera et al., 1997).

In this work we study the possible role of His 186, included in the α 185–200 toxin binding determinant. A peptide with the *Torpedo californica* α 173–202 sequence (TcH) was synthesized

and His 186 was ethoxycarbonylated (TcH-E) or substituted by Ala (TcA). Oxidation with thallium trifluoroacetate was performed in order to establish the α 192–193 vicinal disulfide bridge. Effects of the modifications were evaluated by: a) a solid phase assay; and b) a competition assay between each peptide and the *Torpedo californica* receptor, in solution.

Peptide ethoxycarbonylation and the substitution of His 186 by Ala led to an α -BgTx binding decrease of 60 and 90%, respectively, in dot assays. IC₅₀ values, calculated from the competition assays, were $(3.94 \pm 0.82) \cdot 10^{-6}$ M, $(2.25 \pm 0.73) \cdot 10^{-5}$ M and $(1.48 \pm 0.41) \cdot 10^{-4}$ M, for TcH, TcH-E and TcA respectively. Fourth derivative spectra of native and modified peptides were superimposable thus indicating the absence of any conformational change responsible for the lack of toxin binding.

It should be pointed out that the α 173–202 peptide binds the toxin even though the 192–193 disulfide bridge is reduced. However, the effect of both modifications occurs only when the bridge is present.

Results lead to the conclusion that peptide behaviour depends on the oxidation state of cysteines 192 and 193 and that participation of His 186 in the receptor-toxin interaction requires the existence of the disulfide bridge, as is the case in the native receptor.

References

- Conti-Tronconi, BM, McLane, K, Raftery, M, Grando, S, Protti, M (1994) The nicotinic acetylcholine receptor: structure and autoimmune pathology. *Crit. Rev. Biochem. Mol. Biol.* 29, 69–123.
 Lacorazza, H, Otero de Bengtsson, M, Biscoglio de Jiménez Bonino, M (1992) Role of histidine in the α -bungarotoxin binding of the nicotinic acetylcholine receptor. *Neurochem. Int.* 20, 521–527.
 Lacorazza, H, Otero de Bengtsson, M, Biscoglio de Jiménez Bonino, M (1996a) Key histidine residues in the nicotinic acetylcholine receptor. *Neurochem. Int.* 28, 77–97.
 Lacorazza, H, López, R, Venera, GD, Biscoglio de Jiménez Bonino, M (1996b) Localization of histidine residues relevant for the binding of α -bungarotoxin to the acetylcholine receptor α -subunit in v8 proteolytic fragments. *Neurochem. Int.* 28, 557–567.
 Venera, GD, Testai, FD, Peña, C, Biscoglio de Jiménez Bonino, M (1997) Involvement of Histidine 134 in the binding of α -bungarotoxin to the nicotinic acetylcholine receptor. *Neurochem. Int.* 31, 151–157.

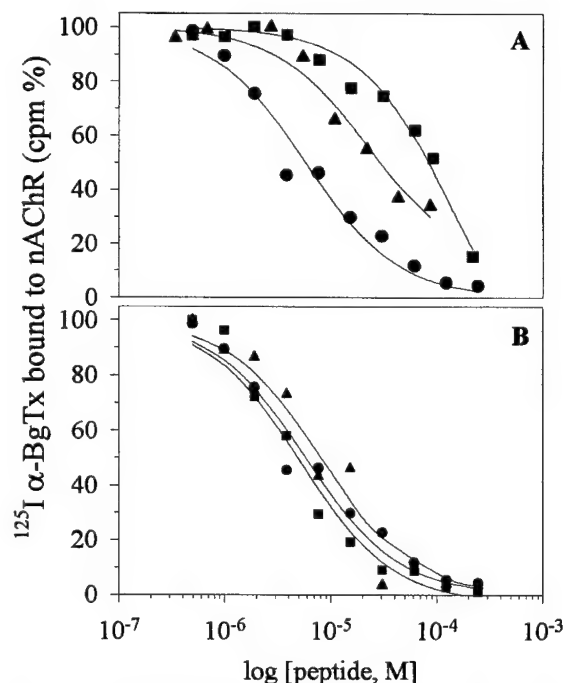


Figure 1. Competition of *Torpedo californica* peptides TcH (●), TcH-E (▲) and TcA (■) for 125 I α -BgTx. Peptides with (A) and without (B) the 192–193 disulfide bridge established, were incubated overnight at 4 °C with 2 pmol of 125 I α -BgTx prior to the addition of 1 pmol of *Torpedo californica* receptor.

108

Effect of nerve growth factor and other differentiating agents on expression of enzymes of acetyl-CoA and acetylcholine metabolism in SN56 cholinergic cells

M. Tomaszewicz^a, A. Jankowska^a, B. Madziar^a, J.K. Blusztajn^b, A. Szutowicz^a

^aDepartment of Clinical Biochemistry, Medical University of Gdańsk, Dębinki 7, 80-211 Gdańsk, Poland

^bDepartment of Pathology, Boston University School of Medicine, Boston, MA, USA

Provision of acetyl-CoA from mitochondria to cytoplasm has been found to play a key role in regulation of acetylcholine (ACh) release and synthesis in depolarization-activated cholinergic neurons. We suppose that competition between demand for acetyl-CoA for energy production and for transmitter synthesis may be one of the sources of preferential impairment of cholinergic neurons in course of several encephalopathies (Szutowicz et al., 1996). However, various populations of cholinergic neurons in the brain display distinct individual differences in their vulnerability to particular degenerative conditions. It may be due to different ratios between their capacities to synthesize acetyl-CoA and to distribute it for energy production and ACh synthesis (Szutowicz et al., 1996). Therefore, in this study we aimed to demonstrate that treatment of one line of septal cholinergic neurons with various differentiating factors is capable to generate several cell phenotypes of different expression of enzymes of acetyl-CoA and ACh metabolism.

Hybrid cholinergic neurons SN56.B5.G4 from mouse septum were grown in culture for 3 days in Dulbecco's modified Eagle's medium containing fetal bovine serum supplemented with differentiating compounds at 37 °C in atmosphere 95% air and 5% CO₂.

Nerve growth factor (NGF) is known to exert differentiating and protective influence on brain cholinergic neurons through its high affinity trkA receptors (trkAR) (Lindsay et al., 1994). We did not find trkAR in SN56 cells. Immunofixation study revealed presence of low affinity p75 binding sites of yet unknown significance. Addition of 1 to 100 ng/mL NGF to culture medium caused concentration-independent inhibition of pyruvate dehydrogenase (PDH) and ATP-citrate lyase (ACL) activities up to 40% and 30%, respectively (figure 1). Activities of choline acetyltrans-

ferase (ChAT) and acetylcholinesterase (AChE) were not affected (figure 2). Adenylate cyclase system has been found to cause differentiation of cholinergic neurons (Berse and Blusztajn, 1997, Szutowicz et al., 1983). Addition of 1 mM dibutyryl cAMP (dbcAMP) resulted in 110% and 37% increases in ChAT and ACL as well as 29% inhibition of PDH activity, respectively. NGF (100 ng/mL) modified none of effects of dbcAMP (figures 1, 2). Stimulation of transcription factor by 0.001 mM all-trans-retinoic acid (RA) (Berse and Blusztajn, 1997) brought about 145% increase of ChAT and 15 and 20% suppression of ACL and AChE activities, respectively. PDH activity did not change. In these conditions NGF caused 50% inhibition of PDH activity and partially reversed inhibitory influence of RA on ACL and its activatory effect on ChAT activity (figures 1, 2). RA and dbcAMP used together had an additive effect on ChAT activity which rose up to 405% of control value. On the other hand, RA partially reversed activatory effect of dbcAMP on ACL. RA did not affect inhibitory and activatory influences of dbcAMP on PDH and AChE activities, respectively. Addition of NGF to the medium containing RA and dbcAMP caused inhibition of ACL and PDH but no modification in expression of cholinergic phenotype (figures 1, 2).

L-carnitine was found to enhance functions of cholinergic neurons both in vivo and in culture presumably by improvement of acetyl-CoA supply to cytoplasmic compartment (Nalecz and Nalecz, 1996). In SN56 cells, it caused concentration-dependent increases of PDH, ACL, ChAT and AChE activities. The highest 72% and 47% rises of ChAT and ACL activities took place at 0.1 mM L-carnitine. The increase of L-carnitine concentration caused gra-

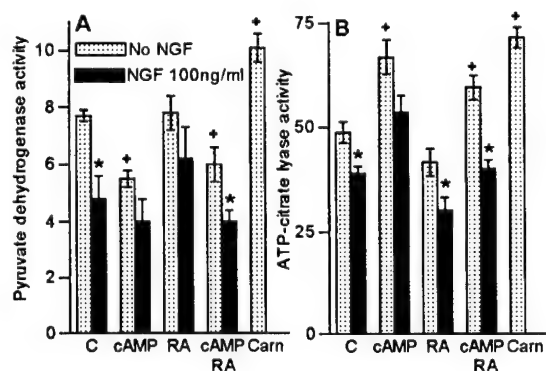


Figure 1. Effect of NGF on specific activities (nmol/min/mg of protein) of PDH (A) and ACL (B) in SN56 cells non-differentiated (C) or differentiated with 1 mM db-cAMP (cAMP) and 0.001 mM retinoic acid (RA). Effect of 0.1 mM (ACL) or 1.0 mM L-carnitine (Carn). Results are means \pm S.E.M. of 6–12 experiments. * $P < 0.05$ compared with respective no NGF conditions; + $P < 0.05$ compared with respective control (C).

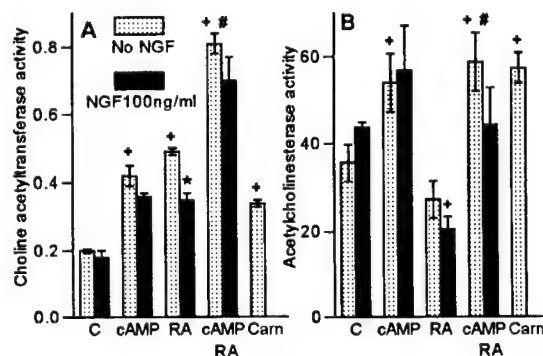


Figure 2. Effect of NGF on specific activities (nmol/min/mg of protein) of ChAT (A) and AChE (B) in SN56 cells non-differentiated (C) or differentiated with 1 mM db-cAMP (cAMP) and 0.001 mM retinoic acid (RA). Effect of 0.1 mM (ChAT) or 0.25 mM (AChE) L-carnitine (Carn). Results are means \pm S.E.M. of 6–12 experiments. * $P < 0.05$ compared with respective no NGF conditions; + $P < 0.001$ compared with respective control (C) for ChAT and $P < 0.05$ for AChE; * $P < 0.005$ compared with respective cAMP or RA conditions.

dual disappearance of this activatory effect. On the other hand, the highest activation of AChE (61%) was observed at 0.25 mM, whereas in the PDH (32%) it occurred at 1.0 mM L-carnitine concentration in the medium (figures 1, 2).

Presented data demonstrate that one parental cholinergic cell may differentiate to various phenotypes with different ratios between acetyl-CoA and acetylcholine metabolism. It indicates that activation of p75 receptors by NGF did not influence development of cholinergic phenotype in SN56 cells. On the other hand, NGF presumably reduced capacity of neurons to generate acetyl-CoA due to suppression of PDH activity. This finding is in agreement with data showing apoptotic effects of p75 receptor activation (Bunone et al., 1997). It may also explain increased vulnerability of NGF-treated cells to neurotoxic inputs. Data presented here demonstrate that development of cholinergic phenotype in cholinergic neurons may be regulated independently by different signal transduction pathways including adenylate cyclase and RA-dependent transcription factor and presumably by L-carnitine mediated stimuli. Changes in the expression of enzymes involved in acetyl-CoA metabolism were independent on cholinergic differentiation since the decrease, instead of expected rise, in PDH and ACL activities was observed after addition of dbcAMP and RA. These results are discrepant with observations made on cAMP-differentiated S-20 neuroblastoma cell and on developing brain where a rise in ChAT was accompanied by the increase in PDH and ACL activities (Szutowicz et al., 1980, 1983). It may be the specific property of SN56 cell line. Nevertheless, relatively low PDH activity in neurons with markedly activated cholinergic metabolism can make them particularly susceptible to different neu-

rodegenerative conditions. On the other hand, effects of L-carnitine on SN56 are in accord with earlier findings showing correlated modifications of ACh and acetyl-CoA metabolism in cholinergic neurons (Nalecz and Nalecz, 1996). Thus, L-carnitine could exert a protective effect on highly differentiated cholinergic neurons by the increase of acetyl-CoA production from pyruvate. This work was supported by K.B.N. project No 4 P05A 04412.

References

- Berse B, Blusztajn JK (1997) Modulation of cholinergic locus expression by glucocorticoids and retinoic acid is cell-type specific. *FEBS Lett.* 410, 175-179.
- Bunone G, Mariotti A, Compagni A, Morandi E, Della-Valle G (1997) Induction of apoptosis by p75 neurotrophin receptor in human neuroblastoma cells. *Oncogene* 14, 1463-1470.
- Lindsay RM, Wiegand SJ, Altar CA, DiStefano PS (1994) Neurotrophic factors: from molecule to man. *Trends Neurosci.* 17, 182-190.
- Nalecz KA, Nalecz MJ (1996) Carnitine - a known compound, a novel function in neural cells. *Acta Neurobiol. Exp.* 56, 597-609.
- Szutowicz A, Kabata J, Lysiak W (1980) ATP-citrate lyase and other enzymes of acetyl-CoA metabolism in developing rat cerebellum and cerebellum. *Int. J. Biochem.* 13, 545-549.
- Szutowicz A, Morrison MR, Srere PA (1983) The enzymes of acetyl-CoA metabolism in differentiating cholinergic (S-20) and non-cholinergic (NIE-115) neuroblastoma cells. *J. Neurochem.* 40, 1664-1670.
- Szutowicz A, Tomaszewicz M, Bielarczyk H (1996) Disturbances of acetyl-CoA energy and acetylcholine metabolism in some encephalopathies. *Acta Neurobiol. Exp.* 56, 323-339.

109

The signaling pathway of calcitonin gene-related peptide-induced acetylcholinesterase expression in muscle is mediated by cyclic AMP

K.W.K. Tsim

Department of Biology and Biotechnology Research Institute, Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong, China

Calcitonin gene-related peptide (CGRP), a neuropeptide synthesized by motor neurons, is able to stimulate the expression of acetylcholine receptor (AChR) and acetylcholinesterase (AChE) that forms the post-synaptic apparatus at the vertebrate neuromuscular junctions (Fontaine et al., 1987; Massoulié et al., 1993). Several lines of evidence indicate that the CGRP-induced AChE expression in muscle is mediated by a G-protein and acted through a cAMP pathway (Choi et al., 1996, 1998). First, various drugs affecting the intracellular level of cAMP, such as N^6, O^2 -dibutyryl adenosine 3', 5'-cyclic monophosphate, cholera toxin, and forskolin, could mimic the effect of CGRP in stimulating the expression of AChE in cultured myotubes. Second, when myotubes were transfected in vitro and in vivo with cDNA encoding constitutively active mutant G_{α_s} , the intracellular cAMP synthesis was increased. The increase in cAMP level was paralleled with an increase in the expression of AChE while the transfection of active mutant G_{α_i} cDNA decreased the cAMP level as well as the AChE expression. Third, the intracellular cAMP-dependent protein kinase (PKA) activity was increased by the application of CGRP while the CGRP-induced AChE expression could be blocked by pre-incubating the myotubes with PKA inhibitors. Last, the chimeric constitutively active ATF1 cDNA when overexpressed in cultured myotubes could stimulate the expression of AChE. Although the mRNA and protein levels of AChE

were increased by CGRP, the enzymatic activity of the CGRP-induced AChE remained relatively unchanged. These findings indicated that the CGRP-induced AChE regulation is mediated by an intracellular cAMP pathway and represented the first evidence to suggest the mRNA synthesis of AChR and AChE could be mediated by the same neuron-derived factor.

References

- Choi, RCY, Leung, PWY, Dong, TTX, Wan, DCC and Tsim, KWK (1996) Calcitonin gene-related peptide increases the expression of acetylcholinesterase in cultured chick myotubes. *Neurosci. Lett.* 217, 165-168.
- Choi, RCY, Yung, LY, Dong, TTX, Wan, DCC, Wong, YH and Tsim, KWK (1998) The calcitonin gene-related peptide-induced acetylcholinesterase synthesis in cultured chick myotubes is mediated by cyclic AMP. *J. Neurochem.* 71, 152-160.
- Fontaine, B, Klarsfeld, A and Changeux, JP (1987) Calcitonin gene-related peptide and muscle activity regulate acetylcholine receptors α -subunit mRNA levels by distinct intracellular pathways. *J. Cell Biol.* 105, 1337-1342.
- Massoulié, J, Pezzementi, L, Bon, S, Krejci, E and Vallette, F-M (1993) Molecular and cellular biology of cholinesterases. *Prog. Neurobiol.* 41, 31-91.

110 'Vital' histoenzymatic reaction and electron microscopic observation of acetylcholinesterase activity in frog neuromuscular junction

S. Tsuji^a, K. Hirai^b, I. Motelica-Heino^a, Y. Katayama^b

^aInstitut des Neurosciences, CNRS-URA 1488, Université de Paris VI, 75005 Paris, France

^bMedical Research Institute, Tokyo Medical and Dental University, Chiyodaku, Tokyo 101, Japan

Acetylcholinesterase (AChE) hydrolyses acetylcholine released in the synaptic cleft. Fine localization of AChE contributes to understanding synaptic functions. In the first step of the present work, we analyse the reaction mechanism of Koelle's copperthiocholine (1949) and Karnovsky's 'direct coloring' thiocholine (1964) methods, the most widely used histochemical methods devised for localization of AChE activity. Based on this analysis we show that it became possible to perform a histochemical reaction derived from these methods, on living tissues in order to avoid artifacts due to postmortem degradation and fixation. Both methods used acetylthiocholine as substrate. In the cholinergic synapse, acetylthiocholine mimics the action of acetylcholine. Acetylthiocholine after enzymatic hydrolysis produces thiocholine, a strong reducing agent transforming cupric and ferric ions in the vicinity of the enzymatic site, respectively into cuprous and ferrous ions. The mechanisms of the chemical reactions taking place in the two methods were studied by Tsuji (1974). It was found that primary precipitate of Koelle's reaction was cuprous thiocholine (white precipitate), while that of Karnovsky's reaction was composed of cupric ferrocyanide (brown precipitate) and of unexpected cuprous thiocholine, chemically identical with Koelle's precipitate. Furthermore, it was observed that this cuprous thiocholine of Karnovsky's reaction was converted during incubation by potassium ferricyanide of the medium into cuprous ferricyanide (another brown precipitate). Thus, Karnovsky's brown precipitate is finally composed of cupric ferrocyanide and cuprous ferricyanide. Tsuji and Larabi (1983) proposed a hypothesis of intramolecular oxidoreduction of copper and iron ions between the two complex salts.

Hanker et al. (1973) observed that Karnovsky's histochemical precipitate was capable to oxidize diaminobenzidine and found that the precipitate is endowed with a catalytic activity. This catalytic activity was interpreted as 'peroxidase-like' activity by Tago et al. (1986). These authors used highly diluted Karnovsky's medium and obtained a fine reaction product, invisible at the level of optical microscopy. The catalytic activity of the invisible precipitate was revealed in a second step by means of a sensitive diaminobenzidine (DAB) and H₂O₂ procedure. The oxidation of DAB was intensified by NiSO₄. Oxidizing catalytic activities of both cupric ferrocyanide and cuprous ferricyanide were demonstrated in vitro towards DAB alone and DAB plus H₂O₂ (Tsuji, 1998). These catalytic activities were explained by intramolecular oxidoreduction between the two complex salts of copper and iron. Therefore, this catalytic activity of Karnovsky's precipitate was qualified as 'oxidoreductase-like'. Furthermore, a modification of Tago's method, without intensification of DAB precipitate by NiSO₄, was proposed for fine electron microscopic cytochemistry.

To our knowledge, no vital histoenzymatic reaction has been devised for the nervous tissue. Indeed, metallic ions present in histochemical mediums appear to be highly toxic (Cooper et al., 1984), contrarily to organic dyes such as methylene blue (Tsuji and Yokoyama, 1981). The unexpected oxidoreductase-like activity described above permitted to dilute Karnovsky's medium. This gave us the possibility to adapt the histochemical medium

for living tissues. In short, Karnovsky's histochemical medium was dissolved in Ringer solution containing citrate buffer and increased calcium chloride. Citrate buffer was necessary first, to stabilize cupric ions in order to hinder undesirable formation of non-histochemical precipitate of cupric ferricyanide and secondly, to reduce inhibitory effect of cupric ions. However, since citrate buffer captures free calcium ions in the synaptic region, an addition of calcium chloride was necessary. Thus, a compromise between the concentration of citrate buffer and that of calcium chloride is essential for vital histochemical reaction. The muscle activity in response to electrical stimulation of motor nerve was monitored by recording of muscular contraction. The transmission lasted 15 to 20 min until complete blockade. We ended 'vital' histochemical reaction at this moment. At this stage, however, the tissue was still alive. Indeed, the transmission was recovered after washing the preparation with Ringer solution containing citrate buffer and increased calcium chloride.

After the 'vital' histochemical reaction, the tissue was fixed by glutaraldehyde, washed and treated by DAB and hydrogen peroxide. Under optical microscope, staining of motor endplates by oxidized DAB precipitate was controlled. The staining was observed in most, if not all, the endplates in the depth of the tissue. After osmification, osmium black of diaminobenzidine gave fine localization of AChE activity in the synaptic cleft between pre- and postsynaptic membranes under the electron microscope. The present work was supported by a Grant-in-Aid for International Scientific Research (Joint Research) from Japan Ministry of Education. We are grateful to Dr. P. Anglade for critical reading of the manuscript.

References

- Cooper GP, Suszkiw JB, Manalis RS (1984) Heavy metals: effects on synaptic transmission. *Neurotoxicology* 5, 247-266
- Hanker JS, Thornburg LP, Yates PE, Moore HG (1973) The demonstration of cholinesterases by the formation of Hatchett's brown. *Histochemistry* 37, 223-242
- Karnovsky MJ, Roots L (1964) A 'direct-coloring' thiocholine method for cholinesterases. *J Histochem Cytochem* 12, 219-221
- Koelle GB, Friedenwald JS (1949) A histochemical method for localizing cholinesterase activity. *Proc Soc Exp Biol Med* 70, 617-622
- Tago H, Kimura H, Maeda T (1986) Visualization of detailed acetylcholinesterase fiber and neuron staining in rat brain by a sensitive histochemical procedure. *J Histochem Cytochem* 34, 1431-1438
- Tsuji S (1974) On the chemical basis of thiocholine methods for demonstration of acetylcholinesterase activities. *Histochemistry* 42, 99-110
- Tsuji S, Larabi Y (1983) A modification of thiocholine-ferricyanide method of Karnovsky and Roots for localization of acetylcholinesterase activity without interference by Koelle's copper thiocholine iodide precipitate. *Histochemistry* 78, 317-323
- Tsuji S (1998) Electron microscopic localization of acetylcholinesterase activity in the central nervous system: chemical basis of a catalytic activity of Hatchett's brown (cupric ferrocyanide) precipitate revealed by 3, 3'-diaminobenzidine.
- Tsuji S, Yokoyama S (1981) Electron microscopical localization of methylene blue staining in myenteric plexus. *Biomed Res* 2, 693-698

111 The NO-mediated effect of carbachol and glutamate on postdenervation decrease of membrane potential in rat diaphragm is directed towards furosemide-sensitive chloride transport

F. Vyskocil, A.Kh. Urazaev, N.V. Naumenko, E.E. Nikolsky

Institute of Physiology, Academy of Sciences of the Czech Republic, Videnská 1083, 142 20 Prague 4, and Department of Animal Physiology and Developmental Biology, Faculty of Sciences, Charles University, Viničná 4, Prague, Czech Republic and Medical University, Kazan, Russia

The membrane potentials of denervated muscle fibers of the rat diaphragm kept in a tissue culture medium are depolarized by about 8–10 mV (10–12%) within 3 h after denervation (1). This early post-denervation depolarization (EPD) is augmented when inward-directed chloride transport is enhanced by high osmolarity (500 mosmol/L) produced by addition of sucrose or NaCl. The EPD and the effect of hyperosmolarity are effectively prevented by the Cl^- transport inhibitor furosemide (1 ± 10^{-4} M) or by a chloride-free bathing medium. The EPD is also substantially reduced (2–3 mV) when muscle strips are bathed with 1 mM L-glutamate (GLU) which is found in motor nerve endings, or with 5 ± 10^{-8} M carbachol (CCh), which mimics the effect of non-quantally released acetylcholine (ACh) (2,3). The hyperpolarizing effects of GLU and CCh on EPD are not influenced by ouabain, an active sodium transport inhibitor, but are absent when NO-synthase is inhibited by L-nitroarginine methylester (NAME) (4) or when Cl^- transport is augmented by increased osmolarity. It is suggested (4, 5) that the post-denervation cessation of non-quantal ACh release (6) and probably also GLU release from nerve endings leads to the activation of the furosemide-sensitive Cl^- transport in the sarcolemma which is responsible for the early post-denervation depolarization.

References

- 1) Urazaev A.Kh., S.T. Magsumov, G.I. Poletaev, E.E. Nikolsky, and F. Vyskocil, 1995, Muscle NMDA receptors regulate the resting membrane potential through NO-synthase, *Physiol. Res.* 44: 205
- 2) Urazaev A.Kh., N.V. Naumenko, G.I. Poletaev, E.E. Nikolsky and F. Vyskocil, 1996, Nitroprusside decreases the early postdenervation depolarization of diaphragm muscle fibres of the rat, *Eur. J. Pharmacol.* 316:219.
- 3) Urazaev A.Kh., S.T. Magsumov, G.I. Poletaev, E.E. Nikolsky and F. Vyskocil, 1997, Acetylcholine and carbachol prevent muscle depolarization in denervated rat diaphragm, *NeuroReport* 8:403.
- 4) Urazaev A.Kh., N.V. Naumenko, G.I. Poletaev, E.E. Nikolsky, and F. Vyskocil, 1998, The effect of glutamate and inhibitors of NMDA receptors on postdenervation decrease of membrane potential in rat diaphragm, *Molecul. Chem. Neuropathology*, in press.
- 5) Urazaev A.Kh., Naumenko N., Nikolsky E.E., Vyskocil F., 1998, Carnosine and other imidazole-containing compounds enhance the postdenervation depolarization of the rat diaphragm fibres. *Physiol. Research*, in press
- 6) Nikolsky E.E., Zemková H., Voronin V.A., Vyskocil F., 1994, Role of non-quantal acetylcholine release in surplus polarization of the mouse diaphragm fibres at the endplate zone. *Journal of Physiology* 477:497.

112 Acetylcholine induces a greater production of prostacyclin in human pulmonary arteries than in veins

L. Walch, J.P. Gascard, B. Leconte, C. Brink, X. Norel

Centre Chirurgical Marie Lannelongue, CNRS-ERS 566, 133, av. de la Résistance, 92350 Le Plessis Robinson, France

Acetylcholine (ACh) is a more potent relaxant agonist in human pulmonary arteries (HPA) than in veins (HPV) (Walch et al., 1997). Vasodilatation is mediated by the release of endothelium-derived relaxing factor (EDRF; Furchgott and Zawadzki, 1980), identified as nitric oxide (NO; Palmer et al., 1987) and prostacyclin (PGI_2 ; Bunting et al., 1976). The aim of this study was to determine the involvement of PGI_2 in the different effects of ACh in HPA and HPV. To this end, the vasodilatation induced by PGI_2 or ACh and the production of PGI_2 were determined in human pulmonary vascular preparations.

Human lung tissue was obtained from patients who had undergone surgery for lung carcinoma. Arteries and veins were dissected from those macroscopically normal regions of the diseased lung. The vascular preparations cut as rings were set up in organ baths (10 mL) containing Tyrode's solution.

To determine the vasodilatations induced by PGI_2 , the vascular preparations were incubated (30 min) with Bay u3405 (1 μM), a TP-receptor antagonist, to avoid any contractile effect of the prostanoids. After a precontraction with noradrenaline (NA, 10 μM), PGI_2 was applied in a cumulative fashion. The sensitivity of HPA and HPV to PGI_2 was similar (pD_2 value, 6.81 ± 0.20 and 6.72 ± 0.19 for HPA and HPV, respectively; $n = 5$).

The effect of indomethacin (INDO), a cyclooxygenase inhibitor, on the ACh-induced relaxations was investigated in human pulmonary vessels. After an incubation (30 min) in absence or presence of INDO (1.7 μM), the tissues were contracted with noradrenaline (NA, 10 μM) and relaxed by addition of ACh in a cumulative fashion. In HPA treated with INDO, a significant decrease of sensitivity to ACh was observed (pD_2 value, 7.42 ± 0.12 and 6.90 ± 0.21 for control and treated preparations, respectively; $n = 5$; Norel et al., 1996). In contrast, in HPV, INDO did not reduce ACh relaxant potency (pD_2 value, 6.07 ± 0.15 and 5.83 ± 0.24 for control and treated preparations, respectively; $n = 5$).

PGI_2 production was determined after ACh stimulation in human pulmonary vessels by measuring the formation of 6-keto- $\text{PGF}_{1\alpha}$, the stable degradation product of PGI_2 , using an enzyme immuno-assay. The vascular preparation were set up in the organ baths, contracted with NA (10 μM) and subsequently relaxed with ACh (1 or 10 μM). The 6-keto- $\text{PGF}_{1\alpha}$ production was measured in aliquots collected from the bath fluid at the end of 5 min prior to NA challenge (basal) and 5 min following the ACh stimulation. Results are presented in table I. However, no difference between the different vessels was observed in the 6-keto- $\text{PGF}_{1\alpha}$ production for 10 min period after NA stimulation. The

Table I.

	Basal (n = 17)	ACh (1 μ M) (n = 8)	ACh (10 μ M) (n = 9)
HPA	8 \pm 1	126 \pm 19	166 \pm 33
HPV	9 \pm 1	34 \pm 11*	70 \pm 17*

Release of 6-keto-PGF_{1 α} (pg/mg of tissue) by HPA and HPV after stimulation with ACh. Values are means \pm S.E. mean and (n) indicates the number of lung samples used. Results obtained after ACh stimulation were corrected for the 6-keto-PGF_{1 α} production due to NA challenge *P < 0.05 relative to similar data obtained in HPA.

level of 6-keto-PGF_{1 α} production was increased by 2.5-fold when compared with the basal production during the 10 min period before NA challenge (HPA, 16 \pm 2 and HPV, 17 \pm 2 pg/mg of tissue; n = 17).

6-keto-PGF_{1 α} production was also evaluated in HPA and HPV before (basal) and after a stimulation with arachidonic acid (AA; 100 μ M). The vascular preparations were set up in the organ baths and the 6-keto-PGF_{1 α} production in the bath fluid was measured for the 15 min period under basal and stimulated conditions. In HPV, the 6-keto-PGF_{1 α} levels measured were 28 \pm 9 and 145 \pm 19 pg/mg of tissue for basal and after AA challenge, respectively (n = 7) while those obtained in HPA were 28 \pm 3 and 229 \pm 43 pg/mg of tissue for basal and AA stimulated conditions, respectively (n = 7).

These results suggest that the greater sensitivity of HPA versus HPV to the relaxant effect of ACh is not due to a greater sensitivity to the relaxant effect of PGI₂. However, this difference could be partly explain by a greater production of PGI₂ in HPA than in HPV. The greater production of this prostaglandin in HPA than in HPV may be in part due to a greater cyclooxygenase and/or PGI₂ synthase activities.

References

- Bunting S, Gryglewski R, Moncada S, Vane JR (1976) Arterial walls generate from prostaglandin endoperoxides a substance (prostaglandin X) which relaxes strips of mesenteric and coeliac arteries and inhibits platelet aggregation. *Prostaglandins* 12, 897-913
- Furchgott T, Zawadzki, JV (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288, 373-376
- Norel X, Walch L, Costantino M, Labat C, Gorenne I, Dulmet E, Rossi F and Brink C (1996). M1 and M3 muscarinic receptors in human pulmonary arteries. *Br. J. Pharmacol.* 119, 149-157.
- Palmer RMJ, Ferrige AG, Moncada S (1987) Nitric oxide release account for the biological activity of endothelium-derived relaxing factor. *Nature* 327, 524-526
- Walch L, Taisne C, Gascard JP, Nashashibi N, Brink C, Norel X (1997) Cholinesterase activity in human pulmonary arteries and veins. *Br. J. Pharmacol.* 121, 986-990

113

The acetylcholinesterase (AChE) of the cobra *Naja naja oxiana*. Identification of residues involved in insensitivity towards fasciculin

C. Weise^a, C. Bon^b, F. Hucho^a, X. Cousin^{b*}

^aInstitut für Biochemie, Freie Universität Berlin, Thielallee 63, D-14195 Berlin, Germany

^bUnité des Venins, Institut Pasteur, 25, rue du Dr-Roux, F-75015 Paris, France

*Present address: Unité de Différenciation Cellulaire et Croissance, Inra, 2 place P-Viala, F-34060 Montpellier, France

AChEs from the venoms of Elapid snakes have been recently characterised in detail. They present several specific properties when compared to other vertebrate enzymes: Venom AChEs are monomers (1) and their sensitivity towards fasciculin, a peptidic toxin purified from the venom of the mamba *Dendroaspis*, varies greatly depending on the species (2). In particular, AChE from the venom of the cobra *Naja* is almost insensitive to this peptidic inhibitor (with an IC₅₀ value \gg 10⁻⁵ M).

In an earlier paper (2), it had been shown that the low sensitivity to fasciculin of AChE from the venom of the krait *Bungarus fasciatus* (bfAChE) can be attributed to the presence of a methionine residue in position 70 and to a lysine residue in position 285, in place of a tyrosine and an acidic residue, respectively. To investigate the mechanism that explains the extremely high resistance of *Naja* AChE (nnAChE) to fasciculin, we have cloned a cDNA coding for AChE from venom glands of the Central Asian cobra *Naja naja oxiana* (nnAChE). The deduced peptidic sequence is 93% identical to bfAChE. Both enzymes share the features explaining the lower sensitivity of bfAChE to fasciculin (M70, K285), and in addition nnAChE carries two mutations in the peripheral site region (A276, K277), conferring a more positive charge to that region of the molecule.

The modification by site-directed mutagenesis of these residues to their counterparts in *Torpedo* or *Bungarus* AChE greatly increases the sensitivity of nnAChE to fasciculin. We describe a mutant which, at low ionic strength, is about four orders of magnitude more sensitive to fasciculin than wild type nnAChE. This shows that point mutations affecting charges in the peripheral site region have large effect on the sensitivity of AChE to fasciculin.

For this mutant, the inhibition by gallamine is strongly enhanced, whereas the inhibition by propidium and bis-quaternary compounds is almost not affected. This shows that the area around the mutated residues 276 and 277 forms a subsite essential for the binding of gallamine.

References

- (1) Cousin, X., Créminon, C., Grassi, J., Meflah, K., Cornu, G., Saliou, B., Bon, S., Massoulié, J., and Bon, C. (1996) *FEBS Lett.* 387:196-200
- (2) Frobert, Y., Créminon, C., Cousin, X., Rémy, M. H., Chatel, J. M., Bon, S., Bon, C. and Grassi, J. (1997) *Biochim. Biophys. Acta* 1339: 253-267
- (3) Cousin, X., Bon, S., Duval, N., Massoulié, J. and Bon, C. (1996) *J. Biol. Chem.* 271: 15099-15108

114 Comparison of the effects of soman on recovery of brain functional acetylcholinesterase and signs of poisoning in the guinea-pig

J.R. Wetherell^a, M.C. Lintern^b, M.E. Smith^b

^aBiomedical Sciences Dept, CBD, Porton Down, Salisbury, Wiltshire, SP4 0JQ, UK

^bDept Physiology, The Medical School, University of Birmingham, B15 2TT, UK

Organophosphate compounds such as soman produce irreversible inhibition of peripheral and central acetylcholinesterase (AChE), resulting in accumulation of acetylcholine (ACh) and a progression of cholinergic signs of poisoning including tremor, salivation, hypothermia and disturbance of motor activity. Studies in the rat have shown little correlation between the inhibition of total brain AChE and the signs of poisoning at 2–48 h (Jovic, 1974).

AChE exists as multiple molecular forms. In mammalian brain the G4 form is the functionally important form. AChE inhibitors have been shown to differentially inhibit the molecular forms of AChE in rat brain (Ogane et al., 1992). Therefore, it is possible that, following AChE inhibitors, a better correlation might exist between the signs of poisoning and the levels of the functional AChE.

Soman (27 µg/kg) produced the expected progression of signs of poisoning. In the brain areas the G1 activity recovered before that of G4. The maximum inhibition measured in the brain did not always reflect the rate of recovery (figure 1). In the striatum the G4 form was inhibited by 78% at 4 h and by 44% at 24 h with recovery to normal levels by 7 days. However, in the cerebellum, the G4 form was inhibited by 73% at 4 h and by 67% at 24 h with little recovery by 7 days.

It seems likely that inhibited functional AChE may be down-regulated and then replaced via increased assembly of the G1 monomer (Chiappa et al., 1995). It has been proposed that the synthesis of precursor G1 may be controlled by the levels of ACh in the cleft. A previous study with a similar dose of soman, showed that ACh levels were significantly elevated in the striatum but not in the cerebellum (Fosbraey et al., 1990). In the present study, in the striatum, where the levels of ACh are high, AChE recovered relatively quickly, whereas, in the cerebellum where the ACh levels are low, the enzyme recovered at a slow rate even though the levels of AChE are relatively high.

The reason for the differential recovery rate of AChE following soman poisoning is unclear. However, the activity of the functionally important enzyme was still reduced 24 h after soman administration

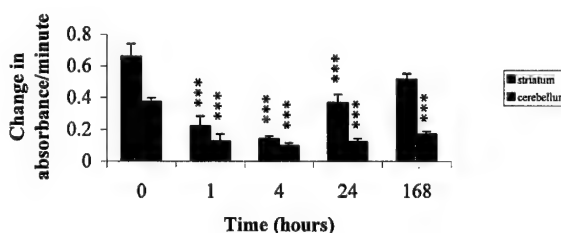


Figure 1. Effect of soman (27 µg/kg) on functional acetylcholinesterase activity in the striatum and cerebellum of the guinea-pig at different time points. The values are the means ± S.E.M. for four animals in each case. ****P* < 0.001 compared to 0 h.

when the overt signs of poisoning had disappeared. It seems likely that there is a mechanism for the early recovery of AChE activity in brain areas known to utilise cholinergic transmission, which facilitates survival following soman poisoning. (c) British Crown Copyright 1998/DERA. Reproduced with the permission of the Controller of Her Britannic Majesty's Stationery Office.

References

- Jovic R C. (1974) Correlation between signs of toxicity and some biochemical changes in rats poisoned by soman. *Journal of Pharmacology* 25,159–164.
- Ogane N, Giacobini E and Messamore E (1992) Preferential inhibition of acetylcholinesterase molecular forms in rat brain. *Neurochemical Research* 17 489–495.
- Chiappa et al. (1995) Slow accumulation of acetylcholinesterases in rat brain during enzyme inhibition by repeated dosing with chlorpyrifos. *Biochemical Pharmacology* 49, 955–963.
- Fosbraey P, Wetherell J R and French MC. (1990) Neurotransmitter changes in guinea-pig brain regions following soman intoxication *Journal of Neurochemistry* 54, 72–79.

115 Alzheimer's disease: Changes in the cerebrocortical expression of nicotinic receptors on the protein level

A. Wevers^a, S. Nowacki^a, J. Lindstrom^b, U. Schütz^a, E. Giacobini^c, H. Schröder^a

^aInstitut II für Anatomie, Universität zu Köln, D-50931 Köln, Germany

^bUniversity of Pennsylvania, School of Medicine, Department of Neuroscience, Philadelphia, PA 19104-6074, USA

^cInstitutions Universitaires de Gériatrie, Université de Genève, CH-1226 Thonex, Switzerland

In cognitive dysfunction syndromes like Alzheimer's disease (AD) impairment of cholinergic transmission and decreased numbers of nicotinic binding sites are well-known features (for review see Schröder and Wevers, 1998). The basic causes of the latter are not well understood and may be due to alterations in the expression

of the nicotinic acetylcholine receptors (nAChR). Previous investigations on the mRNA level have shown that there are no overall alterations in the distribution pattern and in the densities of $\alpha 4$ and $\alpha 7$ transcript expressing neurons in the superior frontal gyrus of AD patients (Wevers et al., 1996). Therefore we now focused

our attention on the level of protein expression of both these subunits. Immunohistochemistry was performed using the subunit-specific antibodies mAb 299 ($\alpha 4$; Peng et al., 1994a) and mAb 306 ($\alpha 7$; Peng et al., 1994b; McLane et al., 1992) followed by the biotin-streptavidin technique. For quantitative analysis camera lucida-drawings were taken to assess the number of nAChR subunit-expressing neurons and the total number of neurons (Nissl-staining). Evaluation was carried out using a two-tailed *t*-test for independent random samples.

In AD there was a marked decrease of labeling intensity for both proteins as compared to controls. Furthermore, in AD the distribution pattern of immunoreactive neurons showed less labeled profiles, especially in the upper cortical layers, than the pattern observed for the neurons expressing the corresponding mRNA, whereas in the control group the patterns of transcript- and protein-bearing neurons looked similar. The quantitative evaluation revealed that the number of $\alpha 4$ and $\alpha 7$ protein expressing neurons was significantly reduced in AD, whereas the total number of neurons was not diminished. These results point to an impaired synthesis of nAChR subunits on the protein level as a possible cause for the cholinergic deficit in AD. Ongoing studies have to elucidate whether the maturation and assembly of receptor protein, the transport and or membrane insertion of the nAChR are

affected. Supported by the Deutsche Forschungsgemeinschaft (Schr 283/18-1, 18-2) and the Stiftung Frau Maria Pesch.

References

- McLane K, Wu X, Lindstrom J, Conti-Tronconi B (1992): Epitope mapping of polyclonal and monoclonal antibodies against two α -bungarotoxin binding subunits from neuronal nicotinic receptors. *J Neuroimmunology* 38, 115–128
- Peng X, Anand R, Whiting P, Lindstrom J (1994a): Nicotine-induced upregulation of neuronal nicotinic receptors results from a disease in the rate of turnover. *Mol Pharmacol* 46, 523–530
- Peng X, Katz M, Gerzanich V, Anand R, Lindstrom J (1994b): Human $\alpha 7$ acetylcholine receptor: cloning of the $\alpha 7$ subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional $\alpha 7$ homomers expressed in *Xenopus* oocytes. *Mol. Pharmacol* 45, 546–554
- Schröder H, Wevers A (1998): Nicotinic acetylcholine receptors in Alzheimer's disease. *Alzheimer's Disease Rev* 3, 21–33
- Wevers A, Monteggia L, Giacobini E, Sullivan J, Maelicke A, Arneric S, Schröder H (1996): Cerebrocortical colocalization of nicotinic receptor $\alpha 4$ - and $\alpha 7$ -subunit mRNA with tau and β -amyloid in M. Alzheimer. *Europ J Neurosci Suppl* 9:147

Immune-mediated slow-channel syndrome: A new form of myasthenia

A.R. Wintzen^a, J.J. Plomp^b, P.C. Molenaar^b, J.G. Van Dijk^a, G.Th.H. Van Kempen^b,
R.M. Vos^c, J.H.J. Wokke^c, A. Vincent^d

^aDept. of Neurology and Clinical Neurophysiology;

^bDivision of Membrane Electrophysiology and Pharmacology, Dept. of Physiology Leiden University Medical Centre, Leiden;

^cDept. of Neurology, University Hospital Utrecht, Utrecht, the Netherlands;

^dNeurosciences Group, Institute of Molecular Medicine, University of Oxford, UK

Autoimmune myasthenia gravis (MG) is caused by antibody mediated loss of functional acetylcholine receptors (AChRs). Here we describe a patient in whom the number of AChRs in the endplate was normal. However, the function of the AChR ion channel appeared to be altered.

We studied the clinical features of the patient, auto-antibodies, electrophysiological features and we did a passive transfer experiment to mice. A 32-year-old female had a 2-year history of diplopia and ptosis, dysphagia and generalised weakness. After rest, the force of the biceps brachii would improve strikingly after 2–3 maximal contractions, after which it decreased quickly. No or borderline level antibodies against AChRs were demonstrated with an immunoprecipitation assay, using denervated human muscle, but an assay using TE 671 cells expressing mainly adult type (AChR- ϵ) was positive (3.5 nmol/L). Pyridostigmine and plasma-

pheresis had no effect. Electromyography revealed decremental response to low frequency (1–5 Hz) but increment at high frequency (10–50 Hz) stimulation. There also were repetitive CMAPs, which decreased with 10 Hz stimulation. Thymectomy revealed a hyperplastic thymus. Electrophysiological study of an intercostal muscle revealed small MEPPs (0.22 ± 0.02 mV, vs. 0.56 ± 0.05 mV in controls) although the number of 125 I- α -bungarotoxin binding sites was normal. The decay time constants of EPPs were prolonged: 23 ± 3.6 ms (controls 5.3 ± 0.6 ms). Ultrastructurally, there was no destruction of the endplate. Injection of the patient's plasma into mice produced similar physiological changes in their diaphragms. We conclude that the patient has an antibody-mediated disorder in which the ion channel properties of the adult form (AChR- ϵ) of the ion channel is changed.

Muscarinic receptors in human melanomas

U. Drews, S. Noda, M. Sailer, M. Oppitz

Institute of Anatomy, University of Tübingen, Österbergstr. 3, D-72074 Tübingen, Germany

In primary and metastatic human melanomas muscarinic cholinergic receptors are present. Muscarinic receptors are expressed, together with embryonic cholinesterase activity, in morphogenetically active embryonic cells. Therefore, the possibility exists, that in melanomas an embryonic trait is re-expressed after transformation. Therefore, we studied the presence of muscarinic receptors in the human melanoma cell line SK-mel-28 by immunofluorescence with the monoclonal antibody M 35 and characterized the receptors further by measuring calcium mobilization after muscarinic stimulation. Cell suspensions were stained with fura-2 and in a fluorimeter cuvette fluorescence was followed at 380 nm excitation. After addition of acetylcholine or carbachol a steep decrease in fluorescence intensity indicated calcium mobilization from intracellular stores (peak reaction), which was followed by constantly lowered fluorescence indicating a steady influx of extracellular calcium in the presence of agonist. By quantitative evaluation dose response curves were obtained, from which a ED of 4.3×10^{-6} M was calculated for acetylcholine and a ED of 2.2×10^{-5} M for carbachol. After pre-incubation with antagonists the dose response curve of acetylcholine was shifted to the right. The inhibition constant of pirenzepine was calculated as 3.9×10^{-7} M, of methoctramine as 6.8×10^{-7} M, and of 4-DAMP-mustard mustard as 1.9×10^{-8} M. Comparison with the data from the literature and those obtained in the chick embryo indicates that the muscarinic receptor in SK-mel-28 melanoma cells pharmacologically behaves as the M3 type and cor-

responds to the embryonic muscarinic receptor characterized by us in earlier studies. In a second experiment, studied the effect of acetylcholine and carbachol on cellular movements in the muscarinic receptor positive human melanoma cell line SK-mel 28 in a perfusion chamber. On perfusion with carbachol and acetylcholine the melanoma cells reacted within 2 to 10 min by concerted cell body contraction and retraction of cell processes. Addition of the muscarinic antagonist atropine to the perfusion medium abrogated the effects of the muscarinic agonists. The experiments demonstrate a direct link between the muscarinic acetylcholine receptor and the contractile apparatus of the melanoma cells. Since melanocytes are derived from neural crest cells which express cholinesterase activity and muscarinic receptors during their migratory phase in the embryo, the re-expression of the muscarinic cholinergic system in melanoma cells may be involved in invasive growth.

References

- Lammerding-Köppel, M., S. Noda, A. Blum, G. Schaumburg-Lever, G. Rassner, U. Drews: Immunohistochemical Localization of Muscarinic Acetylcholine Receptors in primary and metastatic malignant melanomas. *J Cutan Pathol* 25, 137-144 (1997).
- Noda S., M. Lammerding-Köppel, G. Oetting, U. Drews: Characterization of Muscarinic Receptors in the Human Melanoma Cell Line SK-Mel-28 via Calcium Mobilization. *Cancer letters*, in press.

Author index

A

Ackerman E.J., 79, 462, 476
Al-Jafari A.A., 402, 444
Alarcón R., 341, 479
Albrecht C., 257, 445
Albuquerque E.X., 309, 401
Alkondon M., 309, 401
Alliel P.M., 480
Amos M.L., 495
Anand R., 403
Angaut-Petit D., 421
Angus L., 442
Anlauf M., 385
Anselmet A., 183
Antil S., 107, 490
Aparis E., 403
Apel E.D., 167
Araki S., 317
Arce V., 279
Arendt T., 257
Arneric S.P., 221
Arnon R., 501
Arpagaus M., 363
Arroyo-Jimenez M.M., 405, 423
Ashani Y., 461
Augusti-Tocco G., 405
Augustine G., 129

B

Baille V., 369
Bailly L., 177
Balali-Mood M., 375
Balestra B., 434
Ballivet M., 245
Bannon A.W., 221
Bar-On P., 406
Barnéoud P., 418
Bassant M.H., 403
Baubichon D., 453
Beaufort C., 427
Bednar I., 431
Behbehani M.M., 473
Bejanin S., 145
Belkadi L., 421
Benoit E., 456
Bernard V., 407
Berrard S., 145, 408, 478
Berse B., 409, 460
Berti F., 434
Bertrand D., 89, 107, 414, 416, 420, 424, 468
Bertrand S., 414, 416, 420, 424
Besnard F., 418
Besson M.J., 466, 483
Betz H., 129
Biagioni S., 405
Bielarczyk H., 499
Bignami F., 177, 405

Billard J.M., 410
Biscoglio de Jiménez Bonino M., 477, 503
Bitner R.S., 221
Blanchet G., 369
Blandina P., 351
Bloc A., 412
Bloch B., 407
Blusztajn J.K., 199, 409, 413, 441, 460, 504
Bohle R.M., 452
Bohler S., 414, 424
Bon C., 508
Bon S., 183
Bortoletto N., 135
Botti S.A., 414
Boudreau-Larivière C., 442
Bougis P.E., 416, 465
Bourgeois J.P., 405
Bourne Y., 465
Braga M.F.M., 309
Brandeis R., 337
Brandies R., 299
Bren N., 101
Brett C., 385
Briggs C.A., 221
Briley E.M., 431
Brink C., 455, 507
Bruner J., 463
Bugnard E., 412
Buhler C., 357
Buisson B., 89, 416
Burckhart M.-F., 453
Burden S.J., 173
Burgess R.W., 167

C

Camus G., 177
Canini F., 453
Carpentier P., 369, 453
Cartaud J., 177
Cassel J.C., 497
Cermak J.M., 199
Cervini R., 145, 408
Cha N., 475
Chai Y.-G., 448
Champagnat J., 491
Chan R.Y.Y., 442
Changeux J.P., 63, 107, 405, 414, 423, 424, 458, 466, 468, 475, 488, 489
Chapman S., 299
Chapron J., 153, 450
Charbonnier F., 480
Charlton M.P., 129
Charpantier E., 418
Chatonnet A., 419
Chaubourt E., 420

Chen L., 385
Chiamulera C., 225
Chiodini F., 420
Chiou X.G., 454
Chmelar R.S., 275
Cifuentes-Diaz C., 421, 480
Clementi F., 434
Combes D., 363
Cooper S.T., 468
Cordero-Erausquin L.M., 423
Correia-de-Sá P., 423
Corringer P.-J., 107, 414, 424, 458, 466, 468, 490
Cousin X., 419, 508
Couteaux R., 59
Couturier S., 245
Cuello A.C., 205
Culetto E., 363
Curzon P., 221

D

Damaj I., 488
Dani J.A., 209
Davis R.O., 413
De Gois S., 408
De Grandis D., 135
De Jaco A., 405
de Kerchove d'Exaerde A., 489
De La Porte S., 153, 420, 450
Debeir T., 205
DeBello W., 129
Decker M.W., 221
Dedman J.R., 473
deLapeyrière O., 279
Depboylu C., 385
Deprez P., 425
Descaries L., 215
Dettbarn W.-D., 157
Dezaki K., 449
Diebler M.-F., 379
Doctor B.P., 461
Dolezal V., 241, 379, 426
Donnelly-Roberts D.L., 221
Dorandeu F., 369
Drake-Baumann R., 305
Dresbach T., 129
Drews U., 511
Ducancel F., 468
Duclet N., 489
Ducrocq C., 471
Dunant Y., 123, 412
Durkin T.P., 427
Dutar P., 410
Dvorakova M., 452

E

Edelstein S.J., 414, 424
Egorova A., 440

Ehret-Sabatier L., 473
Eiden L.E., 385
Eisenberg O., 337
El Far O., 129
El-Fakahany E.E., 241
Eleopra R., 135
Emerson R.B., 167
Encinar J.A., 432
Enderich J., 257
Engel A.G., 113
Enz A., 406
Erb C., 428
Eric H., 452
Erickson J.D., 141
Eshhar N., 337
Espinosa E., 429
Eusebi F., 434

F

Faille L., 421
Fairén A., 405
Falk-Vairant J., 412
Fauquet M., 478
Favreau P., 430, 456
Fedon Y., 363
Felder C., 414, 431, 454
Feng G., 167
Fernández A.M., 432
Filliat P., 369
Fink L., 452
Fisher A., 299, 337
Fisher J.L., 209
Foquin A., 453
Frieberg L., 431
Friedman A., 329, 446
Frobert Y., 492
Fromm L., 173

G

Garces A., 279
Gascard J.P., 455, 507
Gautam M., 167
Gerzanich V., 403
Giacobini E., 283, 509
Ginsberg D., 489
Gisiger V., 163
Glass D., 167
Goeldner M., 436, 473
González-Ros J.M., 432
Gorbounova O., 438
Gotti C., 434
Goudou D., 421, 480
Grady R.M., 167
Gramolini A.O., 434
Grassi J., 492
Grauso M., 363
Greig N.H., 402
Grubic Z., 435

Grutter T., 436
Gualtieri F., 483

H

Haberberger R., 437, 452
Hamilton S.E., 275
Harel M., 406
Haring R., 337
Harkness P.C., 468
Harlow M., 75
Hashii M., 440
Heilbronn E., 345
Heldman E., 337
Hellström-Lindahl E., 438
Henderson C.E., 279
Henn C., 439
Henrich M., 437
Hernandez M.-C., 245
Hersh L.B., 149, 448
Heuss C., 129
Higashida H., 440
Hirai K., 506
Hoffmann B., 445
Holladay M.W., 221
Holler T., 199
Hoshi N., 440
Hotelier T., 419
Houhou L., 145, 408, 478
Huchet M., 423
Huchet-Dymanus M., 489
Hucho F., 85, 508
Hulme E.C., 269

I-J

Inestrosa N.C., 341, 425, 479
Ishimatsu S., 317
Israël M., 123, 412, 471
Israël M., 379
Jackson D.A., 199
Jakubík J., 241
Jankowska A., 441, 499, 504
Jasmin B.J., 434, 442
Joon J.H., 431
Josse D., 357
Jouvenceau A., 410
Jumel A., 443

K

Kamal M.A., 402, 444
Kanter J., 465, 476
Karczmar A.G., 393
Karton Y., 337
Katayama Y., 506
Katz S., 486
Kaufer D., 329, 446
Kelche C., 497
Keller S.H., 446
Kessler P., 447, 468
Kim M.-H., 448
Kimura I., 449
Klein J., 428, 439
Kline E.F., 470
Koenig J., 153, 450
Korami D., 452
Kostenis E., 265
Koster B., 75
Kotzyba-Hibert F., 436

Kreft S., 435
Krejci E., 167, 183, 425
Krupa A.M., 442
Kryger G., 191
Kummer W., 437
Kummer W., 452
Kuntzweiler T.A., 221

L

Labat C., 455
Lacombe P., 481
Lagadic L., 443
Lallement G., 369, 453, 455
Lam Thanh H., 107
Lamar T.R., 454
Lane L., 471
Lansdell S.J., 468
Laribi O., 407
Lautens L.L., 454
Lawoko G., 345
Lazarus C., 497
Le Gall F., 456
Le Goff A., 492
Le Novère N., 458
Le Sourd A.M., 405
Leblond L., 427
Lecomte M.J., 478
Leconte B., 455, 507
Legall F., 430
Legay C., 183, 457
Léna C., 63, 423
LeNovère N., 423
Letourneux Y., 430, 456
Levey A.I., 407
Lichtman J.W., 167
Lim E.-J., 448
Lindstrom J., 403, 509
Lintern M.C., 459, 495, 509
Lisá V., 426
Livet J., 279
Lockridge O., 357
Loctin F., 412
Löffelholz K., 235, 428, 439
López-Coviella I., 409, 460
Lu J.T., 167
Lu M., 448
Lu Z.-L., 269
Lunde J.A., 434
Luo C., 461

M

Madziar B., 441, 504
Maggi L., 434
Malany S., 79, 462
Mallet J., 145, 408, 478
Malo M., 463
Mankal F.A., 442
Marchand S., 177
Marchot P., 79, 416, 465
Marciano D., 337
Marshall R.M., 75
Martinez K., 466
Marubio L.M., 423
Masson P., 357
Massoulié J., 167, 183, 425, 457, 492
Mathieu-Kia A.M., 466

Matter J.-M., 245
Matter-Sadzinski L., 245
Matz V., 475
Maurin S., 447
Maviel T., 427
Mayhaus M., 257, 445
McArdle J.J., 79, 470
McKinnon L.A., 275
McMahan U.J., 75
Meldolesi J., 119
Menez A., 107, 430, 447, 468, 490
Menini C., 491
Merlo Pich E., 225
Merola F., 466
Meshulam H., 337
Mesulam M.-M., 293
Methfessel C., 85
Meunier F.-M., 379, 475
Meunier F.A., 421
Michaelson D.M., 299
Millar N.S., 468
Millard C.B., 406
Miner J.H., 167
Misawa H., 473
Molenaar P.C., 510
Molgó J., 421, 430, 456
Molles B.E., 79, 470
Montecucco C., 135
Moore D.H., 325
Morel N., 183, 389
Moretti M., 434
Morot Gaudry-Talarmain Y., 471
Moscoso L.M., 167
Moser P., 418
Motelica-Heino I., 506
Mourier G., 468, 490
Mousavi M., 438
Muller D., 420
Murata K., 317

N

Nachon F., 473
Naciff J.M., 473
Narayanan K., 488
Nathanson N.M., 275
Naumenko N.V., 507
Nelson M.E., 403
Nghiem H.-O., 475
Nguyen Q., 167
Nichol M., 167
Nikkel A.L., 221
Nikolsky E.E., 507
Nishikitani M., 317
Nitsch R.M., 257, 445
Noakes P.G., 167
Noda S., 511
Nordberg A., 431, 438
Norel X., 455, 507
Nowacki S., 509

O

O'Regan S., 475
O'Connor V., 129
Oda Y., 145, 408
Ohno K., 113
Okumura T., 317
Ong M.-T., 245

Oppitz M., 511
Osaka H., 462
Osaka H., 79, 476
Otero de Bengtsson M., 477

P-Q

Pajak F., 145, 408, 478
Pallavicini M., 434
Palma E., 434
Parsons I.J., 468
Patrick J., 489
Patrick J.D., 249, 499
Patton B.L., 167
Pavlovsky L., 329, 446
Pellegrini L., 129
Pellizzari R., 135
Peña C., 503
Peng H.B., 195
Pepeu G., 351
Pereira E.F.R., 309, 401
Pérez D., 479
Périn J.-P., 480
Pernot M.I., 455
Peruzzi P., 481
Picciotto M., 488
Pidoplichko V.I., 209
Pittel Z., 337
Plomp J.J., 510
Poindessous-Jazat F.R., 403
Porte S., 420
Poveda J.A., 432
Prado de Carvalho L., 463
Proska J., 482
Puttfarcken P.S., 221
Quiram P.A., 101

R

Ress D., 75
Ribeiro J.A., 423
Rícný J., 483
Rieger F., 421, 480
Rogard M., 466, 483
Romani R., 363
Roshchina V.V., 484
Ross L.S., 454
Rossetto O., 135
Rossi S.G., 195, 486
Rossner S., 257
Rossoni G., 434
Rotundo R.L., 195, 486
Roulet E., 412
Rovati G.E., 434
Roztocil T., 245

S

Sabherwal U., 488
Salmon A., 428, 488
Salmon A.Y., 489
Sanes J.R., 167
Sailer M., 511
Saragovi H.U., 205
Schaeffer L., 489
Schäfer M.K.-H., 385
Schäfer T., 129
Schechtman D., 501
Schlador M.L., 275
Schliebs R., 257

Schmidt B.H., 496
 Schröder H., 509
 Schütz B., 385, 509
 Schwarz M., 75
 Schweizer F., 129
 Seiger Å., 438
 Seil F.J., 305
 Sekine S., 488
 Sendtner M., 490
 Servent D., 107, 430, 468, 490
 Seylaz J., 481
 Sgard F., 418
 Shariat M., 375
 Sharp J.D., 454
 Schiff G., 389
 Shimojo M., 149
 Shoaib M., 229
 Shohami E., 299
 Silman I., 191, 406, 414, 501
 Silva-Barrat C., 491
 Simon S., 183, 492
 Sine S.M., 79, 101, 113, 470, 476
 Slack B.E., 493
 Smith M.E., 459, 495, 509
 Son Y.-J., 167
 Soreq H., 249, 329, 428, 446, 489, 499
 Spencer P.S., 305
 Spowart L., 496
 Sprague D.L., 454

Stemmelin J., 497
 Sternfeld M., 249, 489, 499
 Stetzkowski-Marden F., 177
 Stinnakre J., 463
 Sugiyama N., 79
 Sussman J.L., 191, 406, 414
 Synguelakis M., 389
 Szente M., 491
 Szutowicz A., 441, 499, 504

T

Takasu N., 317
 Tanaka H., 149
 Tarrab-Hazdai R., 501
 Tassonyi E., 420
 Tattersall J.E.H., 502
 Taubenblatt P., 389
 Taupin C., 357
 Taylor P., 79, 416, 446, 462, 465, 470, 476
 Tessari M., 225
 Testai F.D., 503
 Testylier G., 455
 Thevenot E., 408
 Thies R.S., 460
 Timóteo M.A., 423
 Toker L., 501
 Tomasi M., 379
 Tomaszewicz M., 441, 499, 504
 Tonduli L., 455

Toutant J.-P., 363
 Tsigelny I., 79, 476
 Tsim K.W.K., 505
 Tsuji S., 506
 Tucek S., 241, 426, 483
 Tugnoli V., 135

U-V

Urazaez A.Kh., 507
 Urbansky M., 482
 van der Staay F.J., 496
 Van Dijk J.G., 510
 Van Kempen G.Th.H., 510
 Varoqui H., 141
 Velasco E., 421
 Velluti J., 491
 Venera G.D., 503
 Viguie N., 357
 Villa L., 434
 Vincent A., 510
 Vodjdani G., 408
 von der Kammer H., 257, 445
 von Euw D., 481
 Vos R.M., 510
 Vyskocil F., 507

W-Z

Walch L., 455, 507
 Washbourne P., 135
 Watty A., 85

Weber M.J., 429
 Wegner M., 257
 Weihe E., 385
 Weinstock M., 299
 Weise C., 508
 Wells G.B., 403
 Wess J., 265
 Wetherell J., 459, 509
 Wevers A., 509
 Whittaker V.P., 53
 Wikström M.A., 345
 Williams M., 221
 Wintzen A.R., 510
 Wokke J.H.J., 510
 Wood M.W., 431
 Wood S.J., 502
 Wu D., 149
 Wu J., 434
 Yamamoto Y., 279
 Yancopoulos G.D., 167
 Yang Z.P., 157
 Yokoyama K., 317
 Yokoyama S., 440
 Young III W.S., 454
 Younkin S.G., 289
 Zajc-Kreft K., 435
 Zeng F.-Y., 265
 Zhong Z.G., 440
 Zhou H., 167

List of participants

- Edson X. ALBUQUERQUE
Univ. of Maryland - School of Medicine Pharmacology
655 W. Baltimore St.
Baltimore, MD 21201-1156, USA
Tel.: 1 410 706 7333 Fax: 1 410 706 3991
ealbuque@umaryland.edu
- René ANAND
LSU Medical Center - Neuroscience Center
2020 Gravier Street - Suite D
New Orleans, LA 70112, USA
Tel.: 1 504 599 0847 Fax: 1 504 599 0891
ranand@lsu.edu
- Christian ANDRES
Faculté de Médecine - INSERM U316
Bd Tonnelé
37032 Tours, France
Tel.: 33 2 47 36 60 93 Fax: 33 2 47 36 61 52
andres@med.univ-tours.fr
- Alain ANSELMET
Laboratoire de Neurobiologie, CNRS URA 1857
Ecole Normale Supérieure
46, rue d'Ulm
75005 Paris, France
Tel.: 33 1 44 32 37 49 Fax: 33 1 44 32 38 87
- Maria Del Mar ARROYO-JIMENEZ
Institut Pasteur
25, rue du Dr Roux
75015 Paris, France
Tel.: 33 1 45 68 88 44 Fax: 33 1 45 68 88 36
marroyo@pasteur.fr
- Gabriella AUGUSTI-TOCCO
Università "La Sapienza"
Dip. di Biologia Cellulare
Pl A.Moro 5
00185 Roma, Italy
Tel.: 39 649912822 Fax: 39 649912351
tocco@axcasp.caspuir.it
- Mahdi BALALI-MOOD
Imam Reza Hospital
Mashhad University of Medical Sciences
Mashhad, I. R. Iran
- Marc BALLIVET
Université de Genève, Biochimie
30, quai Ernest Ansermet
CH-1211 Genève 4, Switzerland
Tel.: 41 227026494 Fax: 41 227026483
ballivet@scza.unige.ch
- Pazit BAR-ON
Weizmann Institute of Science
Dept of Neurobiology
76100 Rehovot, Israel
Tel.: 972 89343649 Fax: 972 89343141
bnbaron@weizmann.ac.il
- Marie-Hélène BASSANT
INSERM U161 2, rue d'Alésia
75014 Paris, France
Tel.: 33 1 40 78 93 64 Fax: 33 1 45 88 13 04
bassant@broca.inserm.fr
- Dominique BAUBICHON
CRSSA Unité de Neuropharmacologie
38702 La Tronche, France
Tel.: 33 4 76 63 69 55 Fax: 33 4 76 63 69 62
- Véronique BERNARD
Université de Bordeaux II - CNRS UMR 5541
Lab. Histologie-Embryologie
33076 Bordeaux, France
Tel.: 33 5 57 57 15 40 Fax: 33 5 56 98 61 82
Veronique.bernard@umr5541.u-bordeaux2.fr
- Sylvie BERRARD
LGN CNRS UMR C9923
Hôpital Pitié Salpêtrière
83, bd de l'Hôpital
75013 Paris, France
Tel.: 33 1 42 17 75 44 Fax: 33 1 42 17 75 33
sberrard@infobiogen.fr
- Brygida BERSE
Boston University 85 East Newton St. - Room M1006
Boston, MA 02118, USA
Tel.: 1 617 638 5960 Fax: 1 617 638 5400
berse@bu.edu
- Sonia BERTRAND
CMU Dept Physiologie
1, rue Michel Servet
CH-1211 Genève 4, Switzerland
Tel.: 41 227025356 Fax: 41 227025402
- Daniel BERTRAND
Université de Genève
Département de Physiologie C.M.U
1 rue Michel-Servet
CH-1211 Genève 4, Switzerland
Tel.: 41 227025356 Fax: 41 227025402
bertrand@ibm.unige.ch
- Marie-Jo BESSON
Institut des Neurosciences UMR 7624
9, quai Saint Bernard
75005 Paris, France
Tel.: 33 1 44 27 34 00 Fax: 33 1 44 27 26 69
Marie-Jo.Besson@snv.jussieu.fr
- Heinrich BETZ
Max-Planck Institut für Hirnforschung
Abteilung Neurochemie,
Postfach 71 06 62
D-60496 Frankfurt, Germany
Tel.: 49 69 96 769 220 Fax: 49 69 96 769 441
neurochemie@mpih-frankfurt.mpg.de
- Fabrizia BIGNAMI
Institut Jacques Monod
2, place Jussieu
75251 Paris, France
Tel.: 33 1 44 27 42 23 Fax: 33 1 44 27 59 94
bignami@ijm.jussieu.fr
- Jean-Marie BILLARD
INSERM U161
2, rue d'Alésia
75014 Paris, France
Tel.: 33 1 40 78 93 93 Fax: 33 1 45 88 13 04
billard@broca.inserm.fr
- Mirtha Josefa BISCOGLIO
Facultad de Farmacia y Bioquímica
Dept Química Biológica
Junin 956
1113 Buenos Aires, Argentina
Tel.: 54 1 964 82 91 Fax: 54 1 962 54 57
- Jean-Charles BIZOT
Centre d'Etude du Bouchet BP 3
91710 Vert le Petit, France
Tel.: 33 1 69 90 82 67 Fax: 33 1 64 93 52 66
- Anders BJÖRKLUND
Wallenberg Neuroscience Center
Dept of Physiology and Neuroscience
Lund University,
Sölvegatan 17
S-223 62 Lund, Sweden
Tel.: 46 46 222 05 41 Fax: 46 46 222 05 59
anders.bjorklund@mphy.lu.se
- Guy BLANCHET
Direction Centrale du Service de Santé des Armées DCSSA
Sous-Direction AST, Bureau Recherche
rue Saint Dominique
Paris, 00459 Armées
Tel.: 33 1 41 93 26 86 Fax: 33 1 41 28 93 28
- Alain BLOC
Dpt Pharmacology - CMU
1, rue Michel Servet
CH-1211 Genève 4, Switzerland
Tel.: 41 227025436 Fax: 41 227025452
Alain.Bloc@medecine.unige.ch
- Jan Krzysztof BLUSZTAJN
Boston University School of Medicine
85 East Newton St - Room M1009
Boston, MA 02118, USA
Tel.: 1 617 638 4829 Fax: 1 617 638 5400
jbluszt@acs-mail.bu.edu
- Sébastien BOHLER
Institut Pasteur
28, rue du Dr Roux
75724 Paris, France
Tel.: 33 1 40 61 31 03
sbohler@pasteur.fr
- Suzanne BON
Laboratoire de Neurobiologie, CNRS URA 1857
Ecole Normale Supérieure
46, rue d'Ulm
75005 Paris, France
Tel.: 33 1 44 32 38 91 Fax: 33 1 44 32 38 87
- Norbert BONHOMME
Institut de Recherche SERVIE
125, chemin de Ronde
78290 Croissy-sur-Seine, France
Tel.: 33 1 41 18 24 67 Fax: 33 1 41 18 24 60
- Simone BOTTI
Weizmann Institute of Science
Dept. Neurobiology
76100 Rehovot, Israel
Tel.: 972 89343759 Fax: 972 89344159
csbotti@wicc.weizmann.ac.il
- Pierre E. BOUGIS
Faculté de Médecine Nord - CNRS UMR 6560
IFR J Roche, Biochimie
13916 Marseille, France
Tel.: 33 4 91 96 20 70 Fax: 33 4 91 69 88 39
bougis.p@jean-roche.univ-mrs.fr
- Patrick BRETON
Centre d'Etude du Bouchet BP 3
91710 Vert le Petit, France
Tel.: 33 1 69 90 82 76 Fax: 33 1 64 93 52 66
- Clarence A BROOMFIELD
U.S. Army Medical Research Institute of Chemical Defense
1917 Youngston Rd
Jarrettsville, MD 21084, USA
Tel.: 1 410 436 2626 Fax: 1 410 436 1260
Broomfield@asia.apgea.army.mil
- Bruno BUISSON
CMU Dept de Physiologie
1, rue Michel Servet
CH-1211 Genève 4, Switzerland
Tel.: 41 227025356 Fax: 41 227025402
Bruno.Buisson@medecine.unige.ch

- Steven BURDEN**
Molecular Neurobiology Program
Skirball Institute
New York, 10016 NY, USA
Tel.: 1 212 263 7341 Fax: 1 212 263 8215
burden@saturn.med.nyu.edu
- Murielle CARON**
Tre Högarsväg 30A
S-22475 Lund, Sweden
Tel.: 46 121893 Fax: 46 149870
murielle.caron@neurokemi.lu.se
- Jean CARTAUD**
Biologie Supramoléculaire et Cellulaire
Institut Jacques Monod
CNRS, Université Paris 7
2 place Jussieu, 75005 Paris
Tel.: 33 1 44 27 69 40 Fax: 33 1 44 27 59 94
cartaud@ijm.jussieu.fr
- Annie CARTAUD**
Institut J. Monod
2, pl Jussieu
75251 Paris, France
Tel.: 33 1 44 27 42 02 Fax: 33 1 44 27 59 94
cartaud@ijm.jussieu.fr
- Jean-Christophe CASSEL**
LN2C UMR 7521 ULP/CNRS
67000 Strasbourg, France
Tel.: 33 3 88 35 84 35 Fax: 33 3 88 35 84 42
jean-christophe.cassel@psycho-ulp.u-strasbo
urg.fr
- Nicolas CHAMPTIAUX**
Institut Pasteur
28, rue du Dr Roux
75724 Paris, France
Tel.: 33 1 45 68 88 06 Fax: 33 1 45 68 88 36
- Jean-Pierre CHANGEUX**
Neurobiologie Moléculaire, CNRS URA 1284
Institut Pasteur
25 rue du Dr Roux
75015 Paris, France
Tel.: 33 1 45 68 88 45 Fax: 33 1 45 68 88 36
- Eric CHARPANTIER**
Synthelabo Recherche
10, rue des Carrières
92500 Rueil-Malmaison, France
Tel.: 33 1 41 39 13 00 Fax: 33 1 41 39 13 04
- Arnaud CHATONNET**
INRA-DCC
2, place Viala
34060 Montpellier, France
Tel.: 33 4 99 61 23 61 Fax: 33 4 67 54 56 94
chatonne@ensam.inra.fr
- Emmanuel CHAUBOURT**
CNRS UPR 9040
Neurobiologie Cellulaire et Moléculaire
91198 Gif sur Yvette, France
Tel.: 33 1 69 82 36 27 Fax: 33 1 69 82 94 66
chaubou@wat.nbcm.cnrs-gif.fr
- Florence CHIODINI**
CMU, Dept Physiologie
1, rue Michel Servet
CH-1211 Genève 4, Switzerland
Tel.: 41 227025356 Fax: 41 227025402
florence.chiodini@medecine.unige.ch
- Carmen CIFUENTES-DIAZ**
INSERM
17, rue du fer à Moulin
75005 Paris, France
Tel.: 33 1 45 87 61 50 Fax: 33 1 45 87 61 51
cdiaz@infobiogen.fr
- Matilde CORDERO-ERAUSQUIN**
Institut Pasteur - Neurobiologie Moléculaire
25, rue du Dr Roux
75015 Paris, France
Tel.: 33 1 45 68 88 06 Fax: 33 1 45 68 88 36
- Paulo CORREIA-DE-SA**
University Porto Lab Farmacologia - ICBAS
4050 Porto, Portugal
Tel.: 35 12205359 Fax: 35 122001918
farmacol@icbas.up.pt
- Pierre-Jean CORRINGER**
Neurobiologie Moléculaire
CNRS URA 1284
Institut Pasteur
25, rue du Dr Roux
75015 Paris, France
Tel.: 33 1 46 61 31 02 Fax: 33 1 45 68 88 36
pjcorrin@pasteur.fr
- René COUTEAUX**
Laboratoire de Cytologie
7 quai Saint Bernard
75005 Paris, France
Tel.: 33 1 44 27 39 28 Fax: 33 1 44 27 25 08
- Claudio A. CUELLO**
McGill University Pharmacology
3655 Drummond street
H3G 1Y6 Montréal, Canada
Tel.: 1 514 398 3621 Fax: 1 514 398 8317
accuello@pharma.mcgill.ca
- Emmanuel CULETTO**
University of Cambridge
Babraham Institute Laboratory - PO Box 158
CB2 3ES Cambridge, UK
Tel.: 44 1223336600 Fax: 44 1223461954
ec225@mole.bio.cam.ac.uk
- John A. DANI**
Baylor College of Medicine
1 Baylor Plaza
Houston, TX 77030-3498, USA
Tel.: 1 713 798 3710 Fax: 1 713 798 3946
jdani@cns.bcm.tmc.edu
- Michael DECKER**
Dept 47W - AP-GA 100 Abbott Park Rd
Abbott Park, IL 60064-3500, USA
Tel.: 1 847 937 2422 Fax: 1 847 938 0072
michael.decker@abbott.com
- Alban DE KERCHOVE D'EXAERDE**
Neurobiologie Moléculaire
Institut Pasteur
25, rue de Dr Roux
75015 Paris, France
Tel.: 33 1 45 68 88 07 Fax: 33 1 45 68 88 36
albandek@pasteur.fr
- Irudayanadin DELAMANCHE**
Centre d'Etude du Bouchet
BP 3
91710 Vert-le-Petit, France
Tel.: 33 1 69 90 82 58 Fax: 33 1 64 93 52 66
- Sabine DE LA PORTE**
CNRS UPR 9040
Neurobiologie Cellulaire et Moléculaire
91198 Gif sur Yvette, France
Tel.: 33 1 69 82 36 27 Fax: 33 1 69 82 94 66
sporte@wat.nbcm.cnrs-gif.fr
- John R. DEDMAN**
231 Bethesda Ave
Cincinnati, Ohio, USA
Tel.: 1 513 558 4145 Fax: 1 513 558 5738
- Korami DEMBELE**
Faculté Sciences Agronomique de Gembloux
2, passage des Déportés
5030 Gembloux, Belgium
Tel.: 32 81622348 Fax: 32 81622312
dembeler.k@fsagx.ac.be
- Paola DEPREZ**
Universidad Catolica de Chile Neurobiologia
Molecular - Alameda 340 Santiago, Chile
Tel.: 56 2 686 27 23 Fax: 56 2 686 27 17
pdeppez@genes.bio.puc.cl
- Laurent DESCARRIES**
Département de pathologie et biologie cellu-
laire,
Université de Montréal, CP 6128 Succ. Centre-
ville
QC H3C 3J7 Canada
Tel.: 1 515 343 7070 Fax: 1 514 343 5755
descarrl@alife.ere.umontreal.ca
- Wolf D. DETTBARN**
VU Neurology
4422 Wayland Drive
Nashville, TN 37215, USA
Tel.: 1 615 936 2592 Fax: 1 615 936 0223
- Anne DEVILLERS-THIÉRY**
Neurobiologie Moléculaire, CNRS URA 1284
Institut Pasteur
25 rue du Dr Roux
75015 Paris, France
Tel.: 33 1 45 68 88 45 Fax: 33 1 45 68 88 36
devill@pasteur.fr
- Marie-Françoise DIEBLER**
Laboratoire Neurobiologie Cellulaire - CNRS
Av. de la Terrasse
91198 Gif sur Yvette, France
Tel.: 33 1 69 82 36 47 Fax: 33 1 69 82 94 66
diebler@hermes.cnrs-gif.fr
- Bhupendra P. DOCTOR**
Walter Reed Army Institute of Research Divi-
sion of biochemistry DC Washington, DC
20307-5100, USA
Tel.: 1 202 782 3001 Fax: 1 202 782 6304
dr.-Bhupendra-Doctor@wrsmt-pccmail.army
.mil
- Vladimir DOLEZAL**
CNRS - NBCM
Av. de la Terrasse
91198 Gif sur Yvette, France
Tel.: 33 1 69 82 36 71 Fax: 33 1 69 82 94 66
dolezal@wat.nbcm.cnrs-gif.fr
- Ulrich DREWS**
University Tübingen Anatomisches Institut
Osterbergstr. 3
D-72074 Tübingen, Germany
Tel.: 49 70 71 297 2180 Fax: 49 07 07 129 4010
drews@anatom.uni.tuebingen.de
- Yves DUNANT**
Pharmacologie CMU
1211 Genève 4, Switzerland
Tel.: 41 227025432 Fax: 41 227025452
yves.dunant@medecine.unige.ch
- Thomas DURKIN**
Université de Bordeaux II - CNRS UMR 5807
Lab. Neurosciences Comportementales
33405 Talence, France
Tel.: 33 5 56 84 87 45 Fax: 33 5 56 84 87 43
durkin@neurocog.u-bordeaux.fr
- Stuart EDELSTEIN**
Dept Biochimie
30, quai Ernest-Ansermet
CH-1211 Genève 4, Switzerland
Tel.: 41 227026486 Fax: 41 227026476
- Christina ERB**
University of Mainz Dept of Pharmacology
Obere Zahlbacher Str 67
55101 Mainz, Germany
Tel.: 49 61 31 173 173 Fax: 49 61 31 176 611
cerb@mzdmza.zdv.uni-mainz.de

- Jeffrey D. ERICKSON**
Neuroscience Center
School of Medicine in New Orleans
LSUMC,
2020 Gravier Street, Suite "B"
New Orleans, LA 70112-2234, USA
Tel.: 1 504 569 6700 Fax: 1 504 568 5801
jerick@lsu-mc.edu
- Philippe FAVREAU**
Université de La Rochelle Lab. SESNAB
Av Marillac
17042 La Rochelle, France
Tel.: 33 5 46 45 82 27 Fax: 33 5 46 45 82 27
- Christian C. FELDER**
Lilly Research Labs Drop 0510
Lilly Corporate Center
Indianapolis, IN 46285, USA
Tel.: 1 317 276 1380 Fax: 1 317 276 7600
felder@lilly.com
- Abraham FISHER**
Israel Institute Biolog. Research
P. O. Box 19
74100 Ness-Ziona, Israel
Tel.: 972 89381603 Fax: 972 89381615
fisher-a@iibr.gar.il
- Linda FRIBERG**
Huddinge Hospital
Div. Molecular Neuropharmacology
S-14186 Huddinge, Sweden
Tel.: 46 858583899 Fax: 46 858583860
Linda.Friberg@ktcmail.hs.sll.se
- Alon FRIEDMAN**
Dept of Biological Chemistry
The Life Sciences Institute
The Hebrew University
Jerusalem 91904, Israel
Tel.: 972 2 658 5450/658 5109 Fax: 972 2 652 0258
alonf@bgumail.bgu.ac.il
- Wolfram GAIDA**
CNS Research
Boehringer Ingelheim Pharma KG
55216 Ingelheim, Germany
Tel.: 49 61 32 772 098 Fax: 49 61 32 773 848
Gaida@ing.boehringer-ingelheim.com
- Ezio GIACOBINI**
Institutions Universitaires de Gériatrie de Genève
Route de Mon-Idée
1226 Thonex-Genève, Switzerland
Tel.: 41 22 305 65 22 Fax: 41 22 305 61 15
giacobini-ezio@diogenes.heuge.ch
- Victor GISIGER**
Université de Montréal
Département d'Anatomie
Case Postale 6128 - Succursale Centre-ville
H3C3J7 Montréal, Canada
Tel.: 1 514 343 6306 Fax: 1 514 343 2459
gisigerv@magellan.umontreal.ca
- José M. GONZALEZ-ROS**
Universidad Miguel Hernandez Centro de Biología Molecular y Celular E-03206 Elche Alicante, Spain
Tel.: 34 966658757 Fax: 34 966658680
gonzalez.ros@umh.es
- Markus GÖRDES**
Inst. für Anatomie und Zellbiologie
Robert-Koch-Str. 6
D-35037 Marburg, Germany
Tel.: 49 64 21 64000 Fax: 49 64 21 288965
goerdes@mail.uni-marburg.de
- Cecilia GOTTI**
CNR - Center Cellular & Molecular Pharmacology
Via Vanvitelli 32
Milano, Italy
Tel.: 38 270146251 Fax: 38 27490574
gotti@farma4.csfc.mi.cnr.it
- Régis GRAILHE**
Institut Pasteur
Rue du Dr Roux
75015 Paris, France
Tel.: 33 1 45 68 88 46 Fax: 33 1 45 68 88 36
rgrailhe@pasteur.fr
- Anthony O. GRAMOLINI**
University of Ottawa
Medicine Cellular & Molecular Medicine
KIH 8M5 Ottawa, Canada
Tel.: 1 613 562 5800 Fax: 1 618 562 5434
g060109@danis.med.uottawa.ca
- Chris GRANTHAM**
Turnhoutseweg 30
B-2340 Beerse, Belgium
Tel.: 32 14606309 Fax: 32 146111
cgranth@janbe.jnj.com
- Paul GROOT-KORMELINK**
Janssen Research Foundation
Turnhoutseweg 30
B-2340 Beerse Belgium
Tel.: 32 14603965 Fax: 32 14606111
pgrootko@janbe.jnj.com
- Zoran GRUBIC**
Univ. of Ljubljana
Inst. Pathophysiology
Dept Molecular Neurobiology
1000 Ljubljana, Slovenia
Tel.: 386 61310841 Fax: 386 61302272
grubic@ibmi.mf.uni-lj.si
- Thomas GRUTTER**
Faculté de Pharmacie
74, route du Rhin - BP 24
67401 Illkirch, France
Tel.: 33 3 88 67 68 38 Fax: 33 3 88 67 88 91
grutter@bioorga.u-strasbourg.fr
- Christiane GUERIN-STRICKER**
LGME - Institut de Chimie Biologique
11, rue Humann
67085 Strasbourg, France
Tel.: 33 3 88 24 34 61
chrisg@titus.u-strasbg.fr
- Rainer HABERBERGER**
Institut für Anatomie & Zellbiologie
Aulweg 123
D-35385 Giessen, Germany
Tel.: 49 64 19 947 014 Fax: 49 64 19 947 009
rainer.v.haberberger@anatomie.med.uni-gies-sen.de
- Zach HALL**
University of California San Francisco
School of Medicine, Box 410513
Parnassus Avenue
San Francisco, CA 94143, USA
Tel.: 1 415 476 2342
hallz@medsch.ucsf.edu
- Daniel HANTAI**
INSERM U153
Inst. Myologie Hôpital de la Salpêtrière
75013 Paris, France
Tel.: 33 1 42 16 57 06 Fax: 33 1 42 16 57 00
dhantai@myologie.infobiogem.fr
- Edith HEILBRONN**
Stockholm University
Dept Neurochemistry & Neurotoxicology
S-10691 Stockholm, Sweden
Tel.: 46 8164269 Fax: 46 8161371
edith@neurochem.su.se
- Stephen HEINEMANN**
Molecular Neurobiology Laboratory
The Salk Institute
P.O. Box 85800
San Diego, CA 92186-5800, USA
Tel.: 1 619 453 4100 ext 1112 Fax: 1 619 450 2172
heinemann@salk.edu
- Ewa HELLSTRÖM-LINDAHL**
Huddinge Hospital Div. Molecular Neuropharmacology
S-14186 Huddinge, Sweden
Tel.: 46 858583898 Fax: 46 858583860
Ewa.Hellstrom-Lindahl@kfcmail.hs.sll.se
- Christopher HENDERSON**
INSERM U 382
Développement et Pathologie du Motoneurone Spinal
Campus Luminy, Case 907
13288 Marseille cedex 09, France
Tel.: 33 4 91 26 97 66 Fax: 33 4 91 26 97 57
chris@ibdm.univ-mrs.fr
- Claudia HENN**
University Mainz Pharmacologisches Institut
Obere Zahlbacher Strasse 67
D-55101 Mainz, Germany
Tel.: 49 61 31 173 173 Fax: 49 61 31 176 611
cerb&mzdmza.zdv.uni-mainz.de
- Louis B. HERSH**
Chandler Medical Center
Dept of Biochemistry, University of Kentucky
800 Rose Street
Lexington, KY 40536-5549, USA
Tel.: 1 606 323 5549 Fax: 1 606 323 1727
lherhs@pop.uky.edu
- Florence HIBERT**
Faculté de Pharmacie
74, route du Rhin
BP 24
67401 Illkirch, France
Tel.: 33 3 88 67 68 38 Fax: 33 3 88 67 88 91
kotzyba@bioorga.u-strasbourg.fr
- Monique HUCHET**
Neurobiologie Moléculaire, CNRS URA 1284
Institut Pasteur
25, rue du Dr Roux
75015 Paris, France
Tel.: 33 1 40 61 31 13 Fax: 33 1 45 68 88 36
- Ferdinand HUCHO**
Freie Universität Berlin
Institut für Biochemie
Thielallee 63
D-14195 Berlin, Germany
Tel.: 49 30 83 85545 Fax: 49 30 83 83753
hucho@chemie.fu-berlin.de
- Ed C. HULME**
The National Institute of Mental Research
The Ridgeway
NW71AA London UK
Tel.: 44 1819493666
hulme@nimr.mrc.ac.uk
- Carlos IBAÑEZ-MOLINER**
Dept of Med. Chem./Mol. Neurobiol
Karoliska Institute, Box 60400
S-17177 Stockholm, Sweden
Tel.: 46 87287660 Fax: 46 8341960
carlos@cajal.mbb.ki.se
- Nibaldo INESTROSA**
Catholic University of Chile

- Dept of Cell & Molecular Biology
Faculty of Biological Sciences
Alameda 340
Santiago, Chile
Tel.: 56 2 686 27 24 Fax: 56 2 686 27 17
ninstr@genes.bio.puc.cl
- Maurice ISRAEL
Neurobiologie Cellulaire et Moléculaire,
CNRS
91190 Gif sur Yvette, France
Tel.: 33 1 69 82 36 62 Fax: 33 1 69 82 94 66
- Agnieszka JANKOWSKA
Department of Clinical Biochemistry
Medical University of Gdansk
Debinki 7
80-211 Gdansk, Poland
Tel.: 48 58 347 82 22 Fax: 48 58 344 96 53
- Bernard JASMIN
University of Ottawa
451 Smyth Road
K1H 8M5 Ottawa, Ontario Canada
Tel.: 1 613 562 5800 Fax: 1 613 562 5434
bjasmin@danis.med.uottawa.ca
- Mohammad Amjad KAMAL
Dept of Biochemistry
College of Science, King Saud University
P.O. Box 2455
Riyadh 11451, Saudi Arabia
Fax: 966 1 4675791
mamjad@ksu.edu.sa
- Alexander G. KARCZMAR
Hines Hospital Research Service
Hines, Illinois 60191, USA
Tel.: 1 708 343 7200 Fax: 1 708 216 2319
karczmar@research.hines.med.va.gov
- Daniela KAUFER
Hebrew University of Jerusalem
Biological-Chemistry Dept
Life Sciences Institute
91904 Jerusalem, Israel
Tel.: 972 26585454 Fax: 972 26520258
danielak@leonardo.ls.huji.ac.il
- Steve KELLER
University of California San Diego
Dept Pharmacology - 0636
La Jolla, CA 92093, USA
Tel.: 1 619 534 4026 Fax: 1 619 534 8248
skeller@phrtayl@ucsd.edu
- Pascal KESSLER
CEA/SACLAY - DIEP Bât 152
91191 Gif sur Yvette, France
Tel.: 33 1 69 08 52 25 Fax: 33 1 69 08 90 71
pascal.kessler@cea.fr
- Helmut KEWITZ
Kaunstrasse 2
14163 Berlin, Germany
Tel.: 49 30 80 16151 Fax: 49 30 80 16151
- Ikuko KIMURA
Toyama University Dept Chemical Pharma.
2630 Sugitani
930-0194 Toyama, Japan
Tel.: 81 764342281 Fax: 81 764326958
ikukokim@ms.toyama-mpu.ac.jp
- Winifred KOELLE
205 College Avenue
Swarthmore, PA 19081, USA
Tel.: 1 610 544 4566
- Jeanine KOENIG
 Inserm U153
Groupe Hospitalier Pitié Salpêtrière
47, bd de l'Hôpital
- 75651 Paris, France
Tel.: 33 1 42 16 57 05 Fax: 33 1 42 16 57 00
- Eric KREJCI
Laboratoire de Neurobiologie, CNRS URA
1857
Ecole Normale Supérieure
46, rue d'Ulm
75005 Paris, France
Tel.: 33 1 44 32 37 48 Fax: 33 1 44 32 38 87
krejci@biologie.ens.fr
- Wolfgang KUMMER
Institut Anatomie & Zellbiologie
Aulweg 123
35385 Giessen, Germany
Tel.: 49 64 19 947 000 Fax: 49 64 19 947 009
wolfgang.kummer@anatomie.med.uni-giessen.de
- Pierre LACOMBE
CNRS UPR 646 Lab Rech. Cerebrovasculaires
10, av de Verdun
75010 Paris, France
Tel.: 33 1 44 89 77 35 Fax: 33 1 44 89 78 25
lacombe@ext.jussieu.fr
- Herbert LADINSKY
Boehringer-Ingelheim Italia
Via Lorenzini 8
20139 Milano, Italy
Tel.: 39 2 5355258 Fax 39 2 5355205
- Guy LALLEMENT
CRSSA Unité de Neuropharmacologie
B.P. 87
38702 La Tronche, France
Tel.: 33 4 76 63 69 56 Fax: 33 4 76 63 69 62
GuyLallement@compuserve.com
- Tiffanie LAMAR
Lilly Research Labs Drop 0510
Lilly Corporate Center
Indianapolis, IN 46285, USA
Tel.: 1 317 276 9293 Fax: 1 317 276 5546
Lamar-T@Lilly.com
- Lydie LANE
CNRS NBCM
Av. de la Terrasse
91198 Gif sur Yvette, France
Tel.: 33 1 69 82 36 33 Fax: 33 1 69 82 94 66
- Jose A. LASALDE
University of Puerto Rico Dept Biology
JGD 204 - PO Box 23360
00931-3360 Puerto Rico, Puerto Rico
Tel.: 178 77640000 Fax: 178 77640000
joseald@cuqui.net
- Frédéric LE GALL
CNRS UPR 9040 Lab NBCM - Bat 32
91198 Gif sur Yvette, France
Tel.: 33 1 69 82 36 42 Fax: 33 1 69 82 94 66
legall@wat.nbcm.cnrs-gif.fr
- Nicolas LE NOVÈRE
Neurobiologie Moléculaire
Institut Pasteur
25, rue du Dr Roux
75015 Paris, France
Tel.: 33 1 45 68 88 44 Fax: 33 1 45 68 88 36
- Béatrice LECONTE
CCML - CNRS ERS-566 133, av de la Résistance
92350 Le Plessis-Robinson, France
Tel.: 33 1 40 94 82 00 Fax: 33 1 46 30 12 08
brink@pratique.fr
- Claire LEGAY
Laboratoire de Neurobiologie, CNRS URA
1857
Ecole Normale Supérieure
- 46, rue d'Ulm, 75005 Paris, France
Tel.: 33 1 44 32 37 48 Fax: 33 1 44 32 38 87
legay@biologie.ens.fr
- Yves LETOURNEUX
Université de La Rochelle Lab. SESNAB
Av Marillac
17042 La Rochelle, France
Tel.: 33 5 46 45 82 27 Fax: 33 5 46 45 82 27
- Daniel LEVILLAIN
CH du Rouvray Service E.E.G
76301 Sotteville-lès-Rouen, France
Tel.: 33 2 32 95 12 17 Fax: 33 2 32 95 11 77
- Maxine LINTERN
University of Birmingham Dept Physiology
B15 2TT Birmingham, UK
Tel.: 44 1214146897/8 Fax: 44 1214146919
m.c.lintern@bham.ac.uk
- Brian LOCKHART
Institut de Recherche Servier
125, chemin de Ronde
78290 Croissy-sur-Seine, France
Tel.: 33 1 41 18 23 47 Fax: 33 1 41 18 24 60
- Konrad LÖFFELHOLZ
University of Mainz, Pharmacology
Obere Zahlbacher Strasse 67
55101 Mainz, Germany
Tel.: 49 61 31 173 260 Fax: 49 61 31 176 611
LOEFFELH@mzd.mza.zdv.uni-mainz.de
- Ignacio LOPEZ-COVIELLA
Boston University
85 East Newton St. - Room M1009
Boston, MA 02118, USA
Tel.: 1 617 638 4726 Fax: 1 617 638 5400
coviella@bu.edu
- Walter LUYTEN
Turnhoutseweg 30
B-2340 Beerse, Belgium
Tel.: 32 14602618 Fax: 32 14606111
- Jacques MALLET
CNRS UMR 9923
Génétique Moléculaire de la Neurotransmission
et des Processus Dégénératifs LGN
Hôpital de la Pitié Salpêtrière
83 Bd de l'Hôpital
75013 Paris, France
Tel.: 33 1 42 17 75 32 Fax: 33 1 42 17 75 33
- Michel MALO
CNRS UPR 9040 Lab Biologie Cellulaire et Moléculaire
91198 Gif sur Yvette, France
Tel.: 33 1 69 82 36 24 Fax: 33 1 69 82 94 66
Michel.Malo@nbcm.cnrs-gif.fr
- Pascale MARCHOT
Faculté de Médecine - IFR Jean Roche
CNRS UMR 6560 Ingénierie des Protéines
13916 Marseille, France
Tel.: 33 4 91 69 89 02 Fax: 33 4 91 65 75 95
marchot.p@jean-roche.univ-mrs.fr
- Françoise MARDEN
Institut Jacques Monod
2 place Jussieu
75251 Paris, France
Tel.: 33 1 44 27 42 02 Fax: 33 1 44 27 59 94
marden@ijm.jussieu.fr
- Marc MARIEN
Centre de Recherche P. Fabre
Div. Neurobiologie
17, Avenue Jean Moulin
81106 Castres, France
Tel.: 33 5 63 71 42 76 Fax: 33 5 63 71 43 63

- Karen MARTINEZ**
LURE - CNRS CEA et MENESR
Centre Universitaire - Bât 209 D
91405 Orsay, France
- Jean MARY**
Institut Pasteur - Unité des Venins
25, rue du Dr Roux
75015 Paris, France
Tel.: 33 1 45 68 85 79 Fax: 33 1 40 61 30 57
jmary@pasteur.fr
- Patrick MASSON**
CRSSA Dept de Toxicologie
BP 87
38702 La Tronche, France
Tel.: 33 4 76 63 69 59 Fax: 33 4 76 63 69 63
100335.404@CompuServe.com
- Jean MASSOULIÉ**
Laboratoire de Neurobiologie, CNRS URA
1857
Ecole Normale Supérieure
46, rue d'Ulm
75005 Paris, France
Tel.: 33 1 44 32 378 91 Fax: 33 1 44 32 38 87
jean.massoulie@biologie.ens.fr
- Jean-Marc MATTER**
Université de Genève
Dept Biochemistry - Sciences II
CH-1211 Genève, Switzerland
Tel.: 41 227026492 Fax: 41 227026483
jean-marc.matter@biochem.unige.ch
- Lidia MATTER-SADZINSKI**
Université de Genève
Dept of Biochemistry - Sciences II
CH-1211 Genève, Switzerland
Tel.: 41 227026492 Fax: 41 227026483
lidia.matter@biochem.unige.ch
- Jack McMAHAN**
Dept of Neurobiology
Sherman Fairchild Science Building
Stanford University School of Medicine
Stanford, CA 94305-5401, USA
Tel.: 1 650 723 7489 Fax: 1 650 725 3958
- Jacopo MELDOLESI**
DIBIT - Scientific Institute San Raffaele
Via Olgettina 58
20132 Milano, Italy
Tel.: 39 2 2643 2770 Fax: 39 2 2643 4813
meldolj@dibit.hsr.it
- Marina MELONE**
2° Clinica Neurologica
2° Università di Napoli
Piazza Miraglia, 2
80138 Napoli, Italy
Tel.: 39 815665096 Fax: 39 815665096
mmelone@unina.it
- André MÉNEZ**
CEN/SACLAY - DIEP Bât. 142
91191 Gif sur Yvette, France
Tel.: 33 1 69 08 38 03 Fax: 33 1 69 08 90 71
andre.menez@cea.fr
- Emilio MERLO-PICH**
Glaxo Wellcome SpA
Via Alessandro Fleming
37100 Verona, Italy
Tel.: 39 45 92 18 111 or 39 45 58 24 00
emp15105@ggr.co.uk
- Marsel MESULAM**
Northwestern University Medical School
Cognitive Neurology Center
320 East Superior Street, Room 11-450
Chicago, Illinois 60611-3010, USA
Tel.: 1 312 908 9339 Fax: 1 312 908 8789
mmesulam@mwu.edu
- Christoph METHFESSEL**
BAYER AG ZF-FP Biophysik GEB E41
D-51368 Leverkusen, Germany
Tel.: 49 21 43 07876 Fax: 49 21 43 050 698
christoph.methfessel.cm@bayer-ag.de
- Danny MICHAELSON**
George S. Wise Faculty of Life Sciences
Dept of Neurobiology, Tel Aviv University
Ramat Aviv 68878, Israel
Tel.: 972 3 640 9825 Fax: 972 3 640 7643
dmichael@post.tau.ac.il
- Neil MILLAR**
University College London
Dept Pharmacology
WC1E 6BT London, UK
Tel.: 44 1713807241 Fax: 44 1713807245
n.millar@ucl.ac.uk
- Peter MOLENAAR**
Dept Physiology Wassenaarseweg 62
2333 Leiden, The Netherlands
Tel.: 31 715276204 Fax: 31 715276782
p.c.molenaar@physiology.medfac.leidenuniv.nl
- Brian MOLLES**
University of California San Diego
Dept Pharmacology
9500 Gilman drive
La Jolla, CA 92093-0636, USA
Tel.: 1 619 534 4026 Fax: 1 619 534 8248
bmolles@ucsd.edu
- Cesare MONTECUCCO**
University of Padova
Dept. of Biological Sciences
Via Trieste 75
35131 Padova, Italy
cesare@civ.bio.unipd.it
- David H. MOORE**
US Army Medical Research Institute of Chemical Defence
3100 Ricketts Point Road
Aberdeen Proving Ground
Maryland 21010-5425, USA
Tel.: 1 410 671 3278 Fax: 1 410 671 4150
- Nathalie MOREL**
Laboratoire de Neurobiologie, CNRS URA
1857
Ecole Normale Supérieure
46, rue d'Ulm
75005 Paris, France
Tel.: 33 1 44 32 37 48 Fax: 33 1 44 32 38 87
morel@biologie.ens.fr
- Nicolas MOREL**
NBCM - CNRS
Av. de la Terrasse
91198 Gif sur Yvette, France
Tel.: 33 1 69 82 36 47 Fax: 33 1 69 82 94 66
nicolas.morel@cnrs-gif.fr
- Yvette MOROT-GAUDRY**
CNRS NBCM
Av. de la Terrasse
91198 Gif sur Yvette, France
Tel.: 33 1 69 82 36 33 Fax: 33 1 69 82 94 66
morot@hermes.cnrs-gif.fr
- Kim MYUNG-HEE**
University of Kentucky
800 Rose street
Dept of Biochemistry
Lexington, KY 40576-0084, USA
Tel.: 1 606 323 6629 Fax: 1 606 323 1037
mhkim@mailexcite.com
- Florian NACHON**
Faculté de Pharmacie
74, route du Rhin - BP 24
67401 Illkirch, France
Tel.: 33 3 88 67 69 38 Fax: 33 3 88 67 88 91
nachon@bioorga.u-strasbourg.fr
- Jorge M. NACIFF**
231 Bethesda Ave
Cincinnati, Ohio, USA
Tel.: 1 513 558 4141 Fax: 1 513 558 5738
- Neil M. NATHANSON**
Dept of Pharmacology
University of Washington
Box 357750
Seattle, WA 98195-7750, USA
Tel.: 1 206 543 9457 Fax: 1 206 616 4230
nathanso@u.washington.edu
- Patrick NEF**
Hoffmann-La Roche LTP
CH-4070 Basel, Switzerland
Tel.: 41 616810328 Fax: 41 616881780
Patrick.Nef@roche.com
- Hoang NGHIÊM**
Neurobiologie Moléculaire, CNRS URA 1284
Institut Pasteur
24, rue du Dr Roux
75015 Paris, France
Tel.: 33 1 45 68 88 08 Fax: 33 1 45 68 88 36
- Roger M. NITSCH**
Molekular Biologie, Hamburg Univ. UKE
Martinstrasse 52
D-20245 Hamburg, Germany
Tel.: 49 40 47 174 746 Fax: 49 40 47 174 774
nitsch@plexus.uk.uni-hamburg.de
- Xavier NOREL**
CCML - CNRS ERS-566
133, av. de la Résistance
92350 Le Plessis-Robinson, France
Tel.: 33 1 40 94 28 00 Fax: 33 1 46 30 12 08
brink@pratique.fr
- Seana O'REGAN**
Neurobiologie Cellulaire et Moléculaire
CNRS
Av. de la Terrasse
91198 Gif sur Yvette, France
Tel.: 33 1 69 82 36 69 Fax: 33 1 69 82 94 66
oregan@nbcn.cnrs-gif.fr
- Hitoshi OSAKA**
University of California San Diego
Dept Pharmacology - 0636
9500 Gilman drive
La Jolla, CA 92093-0636, USA
Tel.: 1 619 539 1370 Fax: 1 619 539 8248
hitoshi@phrtaylo.ucsd.edu
- Marcela Silvina OTERO DE BENGTTSSON**
Dept de Química Biológica Junin 956-670 piso
1113 Capital Federal, Argentina
Tel.: 54 1 964 82 91 Fax: 54 1 962 54 57
motero@qb.fyb.uba.ar/bengtssop.oriaba.edu.ar
- Yoav PAAS**
Neurobiologie Moléculaire, CNRS URA 1284
Institut Pasteur
25, rue du Dr Roux
75015 Paris, France
Tel.: 33 1 45 68 88 05 Fax: 33 1 45 68 88 36
ypaas@pasteur.fr
- Fabrice PAJAK**
LGN/CERVI Hôpital Pitié Salpêtrière
83 bd de l'Hôpital
75013 Paris, France
Tel.: 33 1 42 17 75 42 Fax: 33 1 42 17 75 33
fpajak@infobiogen.fr

- Lev PAVLOVSKY
Ben Gurion University
Faculty of Physiology
Beer Sheva, Israel
Tel.: 972 76400393 Fax: 972 6400896
pavlovsk@bgumail.bgu.ac.il
- Giancarlo PEPEU
University of Florence Dept Pharmacology
50134 Florence, Italy
Tel.: 39 554271274 Fax: 39 554271280
pepeu@server1.pharm.unifi.it
- Daniel PEREZ
Universidad Catolica de Chile
Neurobiologia Molecular
Santiago, Chile
Tel.: 56 2 686 27 21 Fax: 56 2 686 27 17
dperez@genes.bio.puc.cl
- Jean-Pierre PERIN
INSERM 17, rue du Fer à Moulin
75005 Paris, France
Tel.: 33 1 45 87 61 53 Fax: 33 1 45 87 61 51
- Philippe PERUZZI
CHU Lariboisiere St Louis CNRS UPR 646
Lab. Recherches Cérébrovasculaires
75010 Paris, France
- Lia PRADO DE CARVALHO
Neurobiologie Cellulaire et Moléculaire,
CNRS
Av de la Terrasse
91198 Gif sur Yvette, France
Tel.: 33 1 69 82 36 24 Fax: 33 1 69 82 94 66
prado@nbcn.cnrs-gif.fr
- Jan PROSKA
Brdlikova 287/1d
15000 Prague, Czech Republic
Fax: 42 02 57211863
RCR.C@telecom.cz
- Jan RICNY
Academy of Sciences of the Czech Republic
Institute Physiology - Videnska 1083
14220 Prague, Czech Republic
Tel.: 42 02 4752573 Fax: 42 02 4752488
ricny@biomed.cas.cz
- Monique ROGARD
Institut des Neurosciences UMR 7624
9, Quai Saint Bernard
75005 Paris, France
Tel.: 33 1 44 27 58 45 Fax: 33 1 44 27 26 69
Monique.Rogard@snv.jussieu.fr
- Victoria ROSHCINA
Russian Academy of Sciences
Institute Cell Biophysics
142292 Pushchino, Russia
Tel.: 7 095 9255984 Fax: 7 0967 790509
zoshchina@venus.ibioc.serpukhov.su
- Susana ROSSI
University of Miami
School of Medicine R124
Dept Cell Biology & Anatomy
1600 NW 10th Ave
Miami, FL 33101, USA
Tel.: 1 305 243 6940 Fax: 1 305 545 7166
SROSSI@molbio.med.miami.edu
- Richard ROTUNDO
University of Miami-School Medicine
Dept of Cell Biology & Anatomy R124
Miami, FL 33101, USA
Tel.: 1 305 243 6945 Fax: 1 305 546 7166
rrotundo@molbio.med.miami.edu
- Usha SABHERWAL
AIIMS Dept of Anatomy
110029 New Delhi, India
Tel.: 91 116593245 Fax: 91 116562663
usabeval@medinst.ernet.in
- Anne-Marie SALMON
Neurobiologie Moléculaire, CNRS URA 1284
Institut Pasteur
25, rue de Dr Roux
75724 Paris, France
Tel.: 33 1 45 68 88 08 Fax: 33 1 45 68 88 36
- Asher SALMON
Hebrew University of Jerusalem
Dept Biological Chemistry
Institute of Life Sciences
91904 Jerusalem, Israel
Tel.: 972 26585454 Fax: 972 26520258
asher@leonardo.is.huji.ac.il
- Joshua R. SANES
Washington University Medical School
Dept of Anatomy and Neurobiology
St Louis, MO 63110, USA
Tel.: 1 314 362 2507 Fax: 1 314 747 1150
sanesh@thalamus.wustl.edu
- Laurent SÉGALAT
CNRS-IPMC
660 route des Lucioles
06560 Valbonne, France
Tel.: 33 4 92 95 77 61 Fax: 33 4 93 95 77 08
segalat@unice.fr
- Michael SENDTNER
University of Wurzburg - Dept of Neurology
Josef Schneider Str. 11
97080 Wurzburg, Germany
Tel.: 49 93 12 015 767 Fax: 49 93 12 012 697
sendtner@mail.uni-wuerzburg.de
- Denis SERVENT
CEA/Saclay
Dept d'Ingénierie et d'Etudes des Protéines -
Bât 152
91191 Gif sur Yvette, France
Tel.: 33 1 69 08 52 02 Fax: 33 1 69 08 90 71
denis.servent@cea.fr
- Mohammed SHOAB
Institute of Psychiatry
De Crespigny Park
London SE5 8AF, UK
Tel.: 44 171 919 3324 Fax: 44 171 740 5305
spjumos@iop.bpmf.ac.uk
- Israel SILMAN
Neurobiology, The Weizmann Institute of
Science
76100 Rehovot, Israel
Tel.: 972 89 34 36 49 Fax: 972 89 47 18 49
BNSILM@WEIZMANN.weizmann.ac.il
- Carmen SILVA-BARRAT
46, rue de Montgeron
91800 Brunoy, France
Tel.: 33 1 64 46 68 60 Fax: 33 1 40 77 97 89
silvabar@ccr.jussieu.fr
- Stéphanie SIMON
Laboratoire de Neurobiologie, CNRS URA
1857
Ecole Normale Supérieure
46, rue d'Ulm
75005 Paris, France
Tel.: 33 1 44 32 37 48 Fax: 33 1 44 32 38 87
simon@biologie.ens.fr
- Steven SINE
Mayo Clinic 200
1st Street SW - Rm. 1-135 MSB
Rochester, MN 55905, USA
Tel.: 1 507 284 9404 Fax: 1 507 284 9420
marge@mayo.edu
- Malany SIOBHAN
University of California San Diego
Dept Pharmacology
9500 Gilman Drive - BSB Rm 2039
La Jolla, CA 92093-0636, USA
Tel.: 1 619 534 1370 Fax: 1 619 534 8248
- Barbara SLACK
Boston University 85 East Newton St. - Room
M1007
Boston, MA 02118, USA
Tel.: 1 617 638 5487 Fax: 1 617 638 5400
bslack@bu.edu
- Margaret E. SMITH
University of Birmingham Medical School
Dept Physiology
B15 2TT Birmingham, UK
Tel.: 44 121 414 6903 Fax: 44 121 414 6919
M.E.Smith@bham.ac.uk
- Hermona SOREQ
The Life Sciences Institute
The Hebrew University
Dept of Biological Chemistry
91904 Jerusalem, Israel
Tel.: 972 26585450/6585 Fax: 972 26520258
Soreq@shum.huji.ac.il
- Peter S. SPENCER
Center for Research on Occupational and
Environmental Toxicology
Oregon Health Sciences University
3181 SW Sam Jackson Park Rd.
Portland, OR 97201, USA
Tel.: 1 503 494 4273 Fax: 1 503 494 4278
- Laura SPOWART
Bayer CNS Research, Troponwerke GmbH
Neurather Ring 1
D-51063 Köln, Germany
Tel.: 49 22 16 472 465 Fax: 49 22 16 472 265
- Meira STERNFELD
Hebrew University of Jerusalem
Dept Biological Chemistry
Institute of Life Sciences
91904 Jerusalem, Israel
Tel.: 972 26585468 Fax: 972 26520258
meira@leonardo.is.huji.ac.il
- Andrzej SZUTOWICZ
Medical University of Gdansk
Dept of Clinical Biochemistry
80-211 Gdansk, Poland
Tel.: 48 58 3492775 Fax: 48 58 3449653
aszut@amedec.amg.gda.pl
- Rebecca TARRAB-HAZDAI
Immunology, The Weizmann Institute of
Science
P.O.Box 23
76100 Rehovot, Israel
Tel.: 972 89642657 Fax: 972 89344141
lihazdai@weizmann.il
- Zev TASHMA
Hebrew University School of Pharmacy
Dept Pharmaceutical Chemistry
PO Box 12065
91120 Jerusalem, Israel
Tel.: 972 26758682 Fax: 972 26410740
tashma@mdz.huji.ac.il
- John TATTERSALL
Biomedical Sciences Department CBD
Porton Down
SP4 0JQ Salisbury, UK
Tel.: 44 198 061 3622 Fax: 44 198 061 3741
vjohnt@jeh.tu-net.com
- Palmer TAYLOR
Univ. of California - School of Medicine

- Pharmacology
9500 Gilman Drive
San Diego, La Jolla, CA 92093-0636, USA
Tel.: 1 619 534 1366/402 Fax: 1 619 534 6833
pwtaylor@ucsd.edu
- Fernando D. TESTAI
Dept de Química Biológica Junin 956
1113 Capital Federal, Argentina
Tel.: 54 1 964 82 91 Fax: 54 1 962 54 57
fdtestai@gb.ffyub.uba.ar
- Guy TESTYLIER
CRSSA
38702 La Tronche, France
Tel.: 33 4 76 63 69 73 Fax: 33 4 76 63 69 40
- Maria TOMASZEWICZ
Medical University Gdansk
Dept of Clinical Biochemistry
80-211 Gdansk, Poland
Tel.: 48 58 349 28 85 Fax: 48 58 344 96 53
- Laura TONDULI
CRSSA Centre Emile Parde
BP47
38702 La Tronche, France
Tel.: 33 4 76 63 69 73
- Jean-Pierre TOUTANT
INRA/DCC
2, place Viala
34060 Montpellier, France
Tel.: 33 4 99 61 26 87 Fax: 33 4 67 54 56 94
toutant@ensam.inra.fr
- Victor TSETLIN
Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry
Russian Academy of Sciences
Ul. Miklukho-Maklaya, 16/120
117871, GSP-7, Moscow V-437, Russia
Tel./Fax: 7 095 335 5733
vits@ibchstobc.ras.ru
- Shigeru TSUJI
Inst. Neurosci.- CNRS-URA 1488
7, quai St Bernard
75005 Paris, France
Tel.: 33 1 44 27 36 58 Fax: 33 1 44 27 25 08
shigeru.tsujipsnv.jussieu.fr
- Hiroshi TSUNEKI
Institut Pasteur - Neurobiologie Moléculaire
25, rue du Dr Roux
75724 Paris, France
Tel.: 33 1 45 68 88 08 Fax: 33 1 45 68 88 36
htsuneki@pasteur.fr
- Stanislav TUCEK
Institute Physiology AV CR
Videnska 1083
14220 Prague, Czech Republic
Tel.: 42 02 4752620 Fax: 42 02 4752488
tucek@biomed.cas.cz
- Graciela VENERA
Facultad de Farmacia y Bioquímica
Dept Química Biológica
Junin 956
1113 Buenos Aires, Argentina
venera@mail.etima.ar
- Martine VERDIÈRE-SAHUQUÉ
INSERM U153 - Institut de Myologie
Hôpital de la Salpêtrière
75651 Paris, France
Tel.: 33 1 42 16 57 55 Fax: 33 1 42 16 57 00
mverdier@myologie.infobiogem.fr
- Frantisek VYSKOCIL
Institute of Physiology, CAS
Videnska 1083
Prague 4, Czech Republic
Tel. 42 02 21951111
vyskocil@sun1.biomed.cas.cz
- Heinz VON DER KAMMER
University of Hamburg
Center for Molecular Neurobiology
D-20246 Hamburg, Germany
Tel.: 49 40 47 176 597 Fax: 49 40 47 176 598
kammer@plexus.uke.uni-hamburg.de
- Gerta VRBOVA
University College London
Dept of Anatomy & Developmental Biology
WC1E 6BT London, UK
Tel.: 44 171 419 3383 Fax: 44 171 380 7349
g.vrbova@ucl.ac.uk
- Laurence WALCH
CCML - CNRS ERS-566
133, av de la Résistance
92350 Le Plessis-Robinson, France
Tel.: 33 1 40 94 28 00 Fax: 33 1 46 30 12 08
brink@pratique.fr
- Michel WEBER
Biologie Moléculaire des Eucaryotes - CNRS
118, route de Narbonne
31062 Toulouse, France
Tel.: 33 5 61 33 59 38 Fax: 33 5 61 33 58 86
weber@ibcg.biotoul.fr
- Eberhard WEIHE
Institut f. Anatomy & Cell Biology
Robert-Koch-Str, 6
D-35033 Marburg, Germany
Tel.: 49 64 21 286 247 Fax: 49 64 21 288 965
weihe@mail.uni-marburg.de
- Christoph WEISE
Institut für Biochemie Freie Universität Berlin
Thielallee 63
D-14195 Berlin, Germany
Tel.: 49 30 83 86242 Fax: 49 30 83 83752
dada.chemie.fu-berlin.de
- Jürgen WESS
NIH-NIDDK Bldg 8A - room BIA-05
Bethesda, MD 20892, USA
Tel.: 1 301 402 3589 Fax: 1 301 402 4182
jwess@helix.nih.gov
- Ignaz WESSLER
Universität Mainz - Pharmakologisches Institut
Obere Zahlbacher Str. 67
D-55101 Mainz, Germany
Tel.: 49 61 31 177 398 Fax: 49 61 31 176 611
wessler@mzdmza.zdv.uni-mainz.de
- BHC WESTERINK
Center for Pharmacy, University of Groningen
9713 Groningen, The Netherlands
Tel.: 31 503633307 Fax: 31 503636908
westerink@farm.rug.nl
- Janet WETHERELL
Biomedical Sciences Dept CBD
Porton Down
SP4 0JQ Salisbury, UK
Tel.: 44 198 061 3622 Fax: 44 198 061 3741
- Andrea WEVERS
Universität zu Köln Institut II für Anatomie
J Stelzmann Str 9
D-50931 Köln, Germany
Tel.: 49 22 14 785 957 Fax: 49 22 14 785 318
wevers.anatomie@uni-koeln.de
- Victor P. WHITTAKER
197 Huntington Road
CB3 0DL Cambridge, UK
Tel.: 44 122 327 6922 Fax: 44 122 327 6295
- Jan WILLEMS
Heymans Institute
De Pintelaan 185
B-9000 Gent, Belgium
Tel.: 32 92403919 Fax: 32 92404988
janl.willems@rvg.ac.be
- Kazuhito YOKOYAMA
University of Tokyo Dept Public Health
School of Medicine
113-0033 Tokyo, Japan
Tel.: 81 338164751 Fax: 81 338164751
kashuhito@m.u-tokyo.ac.jp
- Steven YOUNKIN
Mayo Clinic Jacksonville
4500 San Pablo Road
Jacksonville, FL 32224 USA
Tel.: 1 904 953 7353 Fax: 1 904 953 7370
younkin@mayo.edu